ACTA PATHOLOGICA
ET MICROBIOLOGICA
SCANDINAVICA
VOL 69

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

VOL. 69 1967

MUNKSGAARD COPENHAGEN 1967

Munksgaard
International Booksellers and Publishers, Ltd
47, Prags Boulevard, Copenhagen S, Denmark

PRINTED IN DENMARK
VALD PEDERSENS BOGTRYKKERI
COPENHAGEN

INDEX

VOL 69 FASC 1–4 1967

Acta path et microbiol scandinav 69, 1–10 1967

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MAST CELL CHANGES IN INH-INDUCED NEUROPATHY IN THE RAT

By

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Received 22 vi.66

The normal occurrence of mast cells in various compartments of peripheral nerves in several animal species has been clearly demonstrated (*Torp* 1961, *Enerback et al* 1965)

Recent experimental studies have revealed that mast cells in peripheral nerves participate in the tissue reactions occurring in sectioned and crushed peripheral nerves (*Gamble & Goldby* 1961, *Boschi* 1964, *Enerback et al* 1964, 1965, Olsson 1965) At the site of the primary lesion in sectioned and crushed sciatic nerves of the rat we have found an early degranulation of mast cells Furthermore, during the first weeks after nerve section there was a marked increase in the number of endoneurial mast cells throughout the distal degenerating part of the sciatic nerve (*Enerbäck et al* 1964, 1965, *Olsson* 1965)

Neuropathy induced by isonicotinic acid hydrazid (INH) provides an opportunity for further investigations of mast cells in pathological conditions of the peripheral nervous system of the rat Soon after the application of INH in the treatment of tuberculosis, several reports described functional disturbances of peripheral nerves (*Heilmeyer et al* 1952, *Hook* 1953, *Bunger & Schultz Ehlbeck* 1953, *Fust* 1953, *Jones & Jones* 1953 *Lubing* 1953, *Biehl & Nimitz* 1954, *Biehl & Viller* 1954 *Klinghardt et al* 1954 *Oestreicher et al* 1954, *Fust et al* 1955) Morphological lesions of peripheral nerves have also been produced experimentally by feeding laboratory animals with high doses of INH (*Klinghardt* 1954, *Zbinden & Studer* 1955, *Schlaepfer & Hager* 1964 a, b, c) In the recent light and electronmicroscopic investigation by *Schlaepfer & Hager* the peripheral nerve lesions were characterized as a primary axonal degeneration with a subsequent breakdown of the myelin sheaths Later there were regenerative phenomena of axons and myelin sheaths and a mild endoneurial fibrosis However, participation by mast cells in this peripheral neuropathy has not yet been described

The following study was undertaken in order to find out whether the INH induced neuropathy of the rat is associated with mast cell changes in the damaged nerves

MATERIAL AND METHODS

36 Sprague Dawley rats of both sexes were used The rats weighed between 150 and 250 grams at the beginning of the experiments They were kept on a diet of commercial rat pellets and water ad libitum

Under light ether anesthesia, 350 mg/kg body weight isonicotinic acid hydrazide[1] dissolved in Ringer solution was given by oral intubation daily for two weeks, with the exception of one group of rats, which were sacrificed after treatment for one week Control rats received Ringer solution by oral intubation

Groups of three rats were sacrificed by decapitation 1, 3, 4, 6, 10 and 16 weeks respectively after the first dose

The two sciatic nerves were taken for histological examination From rats with observation times of 4, 10 and 16 weeks the following tissues were taken as well dorsal and ventral spinal nerve roots, dorsal root ganglions sciatic plexus, common peroneal nerve, tibial nerve, sural nerve

The tissues were fixed in an acetic acid formalin mixture for 24 hours (*Enerbäck* 1966) Longitudinal paraffin sections, 5 microns thick, from varying levels of the nerves were stained in a 0 5 per cent water solution of toluidine blue in a citric acid disodium phosphate buffer according to McIlwain, with pH adjusted to 4 5 The sections were rapidly passed through alcohol to xylol and mounted in canada balsam

Myelin sheaths were stained by the luxol fast blue, cresyl violet method of *Klüver & Barrera* (1953) and Palmgren s silver impregnation technique was used for axons (*Palmgren 1949*)

RESULTS

All experimental animals showed diminished motor activity during the last days of the treatment but paralyses was never observed There was also a loss of body weight These general signs disappeared rapidly when treatment was suspended

Mast Cells in Nerves of Control Rats

The control rats displayed the same distribution and morphology of mast cells in peripheral nerves as in the normal rats described elsewhere (*Enerback, Olsson & Sourander* 1965) Therefore only a brief summary will be given here

The epineurium and perineurium of all peripheral nerve trunks examined displayed numerous mast cells around blood vessels Scattered mast cells occurred between the nerve fibres in the endoneurium of the nerve fasciculi The endoneurial mast cells were never seen in groups but appeared fairly uniformly distributed in transverse sections of the nerves The cytoplasm was most often elongated along the line of the nerve The cytoplasmic granules of the mast cells showed a strong metachromasia that was resistant to alcohol dehydration in specimens stained with toluidine blue *No mast cells were identified* in dorsal and ventral spinal nerve roots In dorsal root ganglions, mast cells were found in the capsule and occasionally in the ganglion-nerve junction but never within the ganglions

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1 Isoniazid® (INH) obtained from ACO Stockholm Sweden

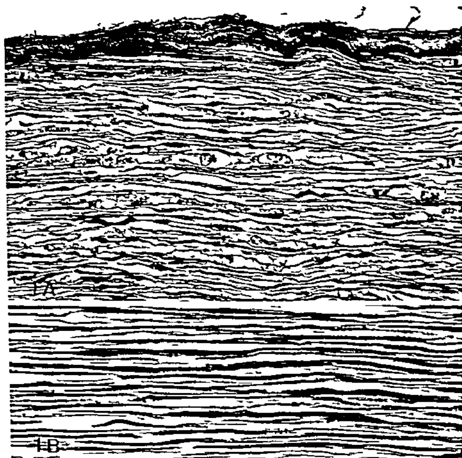


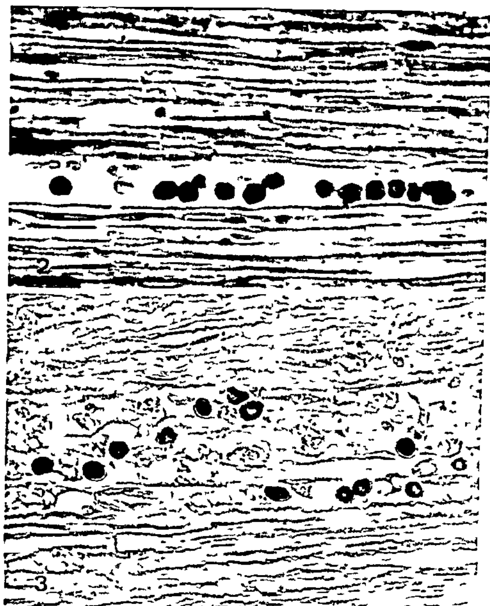
Fig 1

A Marked degenerative changes of axons in the sciatic nerve of a rat treated with INH for fourteen days B Normal appearance of axons from a control rat Silver impregnation (*Palmgren*)

Nerve Fibre Changes in INH-Treated Rats

The earliest changes were observed in axons, after treatment for one week with INH Focal framentations and varicosities of some axis cylinders were found in silver impregnated specimens of the sciatic *nerve in all the rats Such lesions were much more advanced after treat-*ment for 14 days there also seemed to be a marked reduction in the number of axons (Fig 1) In the latest stages however, such axonal changes were much less pronounced

In contrast to the axonal changes observed after treatment for one week, only slight changes of myelin sheaths could be detected in adjacent specimens stained by the luxol fast blue cresyl violet technique of *Klüver & Barrera* These animals displayed only a few varicosities and fragmentations of myelin sheaths After treatment for two weeks,



Figs 2 3

Fig 2 Demyelination and accumulation of mast cells in the demyelinated part of a sciatic nerve from an INH treated rat *Klüter & Barrera*

Fig 3 Focal nerve fibre lesion associated with an accumulation of small rounded endoneurial mast cells in the sciatic nerve of an INH treated rat Phase contrast micrograph of a toluidine blue stained specimen

all rats showed marked demyelination, often with focal areas exhibiting varicosities and fragmentation of the myelin sheaths (Figs 2 3) Rats with observation times exceeding ten weeks presented no clear changes in myelin sheaths of the sciatic nerves

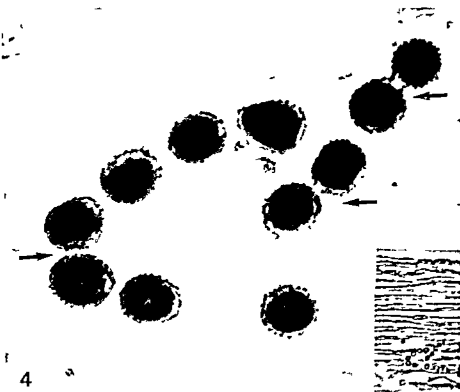


Fig 4

Cluster of mast cells in the vicinity of an endoneurial blood vessel in the sciatic nerve of a rat treated with INH Note the almost identical appearance of the sparsely granulated mast cells and cytoplasmic bridges between adjacent cells (arrows) Toluidine blue

In the damaged areas, mitosis of Schwann cells was occasionally found in the first two groups of rats Later Schwann cells formed so caller Büngner's band in the damaged parts

Changes in axons, myelin sheaths and Schwann cells were most pronounced in the sciatic nerve and its branches, *e g* the common peroneal, tibial and sural nerves In dorsal and ventral nerve roots nerve fibre lesions were extremely rare In dorsal root ganglions, a few ganglion cells displayed tigrolysis of their Nissl bodies and fragmentation of some axis cylinders

Mast Cell Changes in Peripheral Nerves of INH Treated Rats

The earliest morphological changes in mast cells were found in the sciatic nerve of rats given INH for one week Parts in and adjacent to areas showing degenerative changes of nerve fibres had scattered endoneurial mast cells with special morphological characteristics These cells had a rounded nucleus, which occupied the central part of the cell

doxine deficiency (*Oestreicher et al* 1954) There are also observation that deficiency in pyridoxine may be associated with a peripheral neuropathy (*Spies et al* 1939 *Vilter et al* 953 *Swank & Adams* 1945 *Victor & Adams* 1956)

The present findings of endoneurial mast cell changes associated structurally and temporally with degenerative changes of axons and myelin sheaths and the absence of such mast cell changes in the epineurium suggest that this mast cell reaction is secondary to the nerve fibre lesions

It has previously been shown that similar morphological and numerical changes of endoneurial mast cells occur in the distal part of sectioned and crushed nerves of the rat (*Enerback et al* 1964 1965 *Olsson* 1965) The present finding of similar endoneurial mast cell changes in a metabolically induced peripheral neuropathy strengthens the hypothesis that the endoneurial mast cell reaction is elicited from the damaged nerve fibres

SUMMARY

A study was made on the behaviour of mast cells in peripheral nerves in INH induced neuropathy of the rat The main findings were as follows

1 Marked lesions of axons and myelin sheaths in the sciatic nerve andt its main brances In spinal nerve roots nerve fibre lesions were extremely rare

2 In conjunction with degenerative changes of axons and myelin sheaths in the peripheral nerves there was an increasing number of small sparsely granulated mast cells which often appeared in pairs or clusters of cells of almost identical appearance Occasionally small cytoplasmic bridges were detected between adjacent cells In the latest stages the numerous endoneurial mast cells were of about the sam form and size as in control nerves In contrast no mast cell changes were seen in the epineurium Nor could mast cells be found in th spinal nerve roots of INH treated or control rats

These observations suggest that there is a new formation of endoneurial mast cells in this metabolically induced peripheral neuropathy of the rat This finding supports the assumption that the numerical increase of endoneurial mast cells in the distal part of sectioned or crushed nerves is elicited from the degenerated nerve fibres

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Acta path et microbiol scandinav 69, 11–18 1967

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ANTI-NUCLEAR FACTORS IN HEPATIC CIRRHOSIS

With Special Reference to the Different Nuclear Antigenicity of Various Tissues

By

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Received 12 viii 66

Sera from patients with hepatic cirrhosis often contain antibodies against various tissue components, and anti nuclear factors (ANF) as determined by the immunofluorescence technique are found with a positive frequency varying from 12 per cent to 44 per cent (7, 12, 13, 18) These differences are probably mainly due to the composition of the series of patients investigated, but some of them may also arise from the use of different nuclear antigens for the studies Some different ANF reactivities have been reported before Thus, *Hymans et al* (14) suggested the presence of at least two antigen-antibody systems, human sera with a very limited nuclear reactivity have been found by different authors (5, 9, 10 11, 16, 19), tissue-specific ANF has been produced in animals (2, 15), and evidence of granulocyte specific ANF has been presented (2, 3, 8)

When sera from patients with hepatic cirrhosis were examined for the presence of different auto antibodies, it was observed that some sera giving positive reaction for ANF with some tissues as antigen, were completely negative with other tissues Since the variations found did not seem to be explained by technical circumstances only, and the findings might suggest different organ specificity of the anti nuclear factors, it was decided to extend the investigation The present report gives the results of determining anti nuclear factors using the immunofluorescence technique with 8 different sources of nuclei as antigen

MATERIAL

Sera from 96 patients with hepatic cirrhosis collected during the years 1959 63 were investigated All patients showed clinical and biochemical signs of chronic

Part of this study was presented before the 98th meeting of the Danish Society of Pathology Copenhagen Denmark August 25 1965 and before the First Congress of the European Society of Pathology Warsaw June 1 1966
This report was aided by grants from *The Daell Fond* and *Konsul Ehrenfried Queten og hustrus fond*

liver damage In 83 of the cases the diagnosis was confirmed by biopsy or at autopsy The sera that had been stored at 20° C for several years had been used previously for other serological studies (6) Fifity nine of the patients were women 37 were men Twenty six of the female and 5 of the male patients were known to have had epidemic hepatitis Twenty four of the male and 3 of the female patients were known to have been heavy drinkers More than half of the patients were between 50 and 70 years of age Twenty five of the 96 patients had died

Control sera from individuals matched for sex and age were partly derived from healthy donors under 65 years of age (72 sera) and partly from surgical depart ments from patients over 65 years old with a negative Wassermann reaction (24 sera)

METHODS

The two layer indirect immunofluorescence technique was performed as described in a previous paper (7) using undiluted sera

Antigens The following human tissues were employed as antigen *thyroid gland* from a patient with thyrotoxicosis *liver* tissue from a patient with secondary biliary cirrhosis mucosa of the *stomach* from a patient with a duodenal ulcer *striated muscle* from the same patient and normal *renal cortex* adjacent to a renal tumour These tissues were all obtained from the operation theatre cut into pieces and frozen at —70° C within 15 minutes from the time of removal of the tissues the tissues were stored at —70° C until use In addition *blood smears* from peripheral blood of healthy individuals of blood group 0 were dried with a fan and used unfixed within 15 minutes after their preparation Finally the *adrenal gland* from a monkey (guenon) was used as antigen prepared in the same way as de scribed for the human tissues above No fixation was ever used

All pathological sera were investigated at least twice with intervals of weeks or several months using the 6 different human tissues as antigen Discrepant results occurred in few cases Such sera were retested as were sera showing faint or d lt ful nuclear fluorescence until confident results were achieved Appropriate contr l slides without serum and slides with ANF negative and positive control sera were included in all experiments

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conjugate which was absorbed with acetone dried guinea pig liver powder never showed non specific anti nuclear reactivity during daily use for nine months

Microscope

The microscope was a Leitz Ortholux (Zerniche) with equipment for combined fluorescence and phase microscopy with a high pressure mercury vapour lamp (Osram HBO 200) and UG1 exciter filter and a colourless UV absorbing filter

RESULTS

The results are presented in the Tables 1 and 2 and in Fig 1 As seen from Table 1 only 11 of the pathological sera combined with nuclei f striated muscle one fourth of the 96 sera reacted with the nuclei f liver tissues gastric mucosa and those of neutrophilic granulocytes f peripheral blood while about one third of the sera showed a positive reaction for ANF when thyroid gland or small lymphocytes from peripheral blood were used as antigens With monkey adrenal gland almost three fourths of the sera gave positive ANF reaction

Among the control sera none reacted with the nuclei of striated muscle 4 12 sera showed positive ANF reaction with the other human

tissues, while 16 sera combined with the nuclei of monkey adrenal glands

Of the positive reactions with the human tissues 85–90 per cent were given by sera from female patients Thus 17–50 per cent of all female and 3 22 per cent of all male patients contained anti nuclear factors in their sera This preponderance of the female sex was not so distinct when guenon tissue was used as antigen, since two thirds (24 out of 37) of the male as compared to three fourths of the female patients (46 out of 59) showed positive ANF reactions

TABLE 1

Positive Frequency of ANF in 96 Cirrhotic Sera and 96 Control Sera Using 7 Different Human Tissues and One Animal Tissue as Antigen

Number of sera	Human tissues: Striated muscle	Renal cortex	Liver	Gastric mucosa	Neutrophile granulocyt	Thyroid gland	Lymphocytes	Monkey tissue: Adrenal gland
96 patient sera								
Female 59	10	22	23	23	21	31	28	46
Male 37	1	2	2	3	5	4	8	24
96 control sera								
Female 59	0	6	7	4	3	8	4	13
Male 37	0	1	2	2	1	4	1	3

From Fig 1, in which the positive ANF reactions using the different tissues as antigen are placed in groups according to age of patients and controls, it is seen that most of the positive reactions were found in the age groups between 60 and 80 years, regardless of the tissues employed

The varying incidence of ANF found may simply reflect quantitative differences of antigen present in the nuclei, but since the differences in nuclear susceptibility were unrelated to the titres of ANF, it may also be due to the presence of anti-nuclear factors with limited nuclear reactivity, i e organ specific anti-nuclear factors Table 2 shows an analysis with respect to this point Fifteen sera had very limited nuclear reactivity since they reacted with only one of the 7 human nuclear antigens Six of these sera stained the small lymphocytes only, 6 the thyroid nuclei only and 3 the nuclei of the gastric mucosa only No other ANF with apparent organ specificity were found among the pathological sera, except for one serum which reacted with the nuclei of the eosinophilic granulocytes but not with those of neutrophils, lymphocytes or any other of the human nuclei tested One serum showed a very strong fluorescence of the nuclei of the arterial walls in the tissues, besides a very faint staining of the nuclei of gastric mucosa as the only other ANF present in this serum specimen

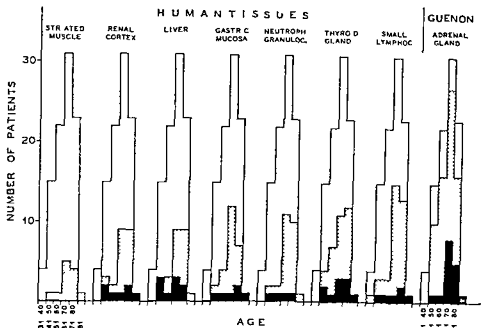


Fig 1

Positivity of ANF in 96 control sera (black columns) and 96 cirrhosis sera (check pattern) in different age groups using 7 human tissues and one animal tissue as antigen

Seven sera contained ANF which reacted with all human tissue nuclei, 28 sera showed positive ANF reaction with more than one but not all human tissues, and altogether 51 (50 + 1) of the pathological sera showed ANF reactivity with one or more human tissues Fourty five (46 — 1) of the pathological sera did not react with any of the 7 different human tissue nuclei

All sera from cirrhotic patients, except one reacting with one or more of the human tissues, also reacted with the nuclei of the monkey adrenal gland Twenty-two of the 46 sera not reacting with any of the

TABLE 2

Number of Hepatic Cirrhosis and Control Sera Reacting with One or More Nuclei of 7 Different Human Tissues Employed as Antigen

	Number of human tissues reacting							Total number of positive ANF with one or more tests	Number of negative sera in all tests
	1/7	2/7	3/7	4/7	5/7	6/7	7/7		
Patient sera 96	15	6	3	5	5	9	7	50(+1)*	46(—1)
Control sera 96	4	3	4	1	1	2	0	15	81

* One serum reacted with the nuclei of eosinophilic granulocytes only

human tissues did not react with the nuclei of the monkey adrenal gland either Twelve of the 15 sera containing apparently organ specific ANF with human tissues gave positive reaction with the nuclei of guenon adrenal gland also

With regard to the control sera, 4 of them reacted with the nuclei of only one tissue, but not with the other 6 human tissues and 11 sera stained the nuclei of more than one tissue None of the control sera *reacted with all nuclei tested Eighty one of the control sera did not* react with any of the human nuclei tested Sixteen of the control sera gave a positive ANF reaction with monkey adrenal gland Among these 16 sera were 12 reacting with one or more of the human tissues and 4 sera showing no other ANF reactivity

DISCUSSION

The present demonstration of dependence of anti nuclear response on the nature of the tissue employed as antigen shows that the choice of nuclear source may be important when testing sera for anti nuclear reactivity This applies to the sera from the patients with hepatic cirrhosis and to those from the present control material as well as to sera from patients with myasthenia gravis, as *Feltkamp* (9) has shown and to sera from patients with rheumatoid arthritis (3), but it is probably of little or no importance with sera from patients with disseminated lupus erythematosus possessing greater reactivity with different tissue nuclei (1) The fact that such sera have been used as positive control sera in most of the reports dealing with determinations of ANF in different pathological states may explain why only few recent papers suggest a possibly different antigenicity of nuclei of various tissues

Though a standardization of all the steps in the technique which is highly necesssary in comparative investigations of this kind has as far as possible been carried out, it is still feasible that unnoticed technical differences (*e g* in preparation storage or handling of tissues or sera with a consequent degradation of antigen or antibody) may explain some of the varying results presented Therefore the findings call for carefull attempts to standardize nuclear antigen in addition to the examinations of the significance in the reaction of the composition of conjugates etc However, the lack of agreement between the reactivity of the different nuclei and the variability in antibody titres besides the identical results repeatedly obtained even with great intervals suggest the existence of antinuclear factors with different organ (and species) specificities It has been an invaluable help to use phase contrast microscopy, and conventional staining of the immuno fluorescence preparations has sometimes also been necessary to ascertain which nuclei actually were stained These procedures have in some cases revealed that fluorescent nuclei in sections from cirrhotic livers originated in infil

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Acta path et microbiol scandinav 69, 19 27 1967

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MICROTECHNIQUE FOR QUANTITATIVE MEASUREMENT OF RENIN IN PLASMA

By

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Received 30 vi 66

Many methods of measuring renin activity have been described in the last few years *Brown et al* (1964), *Boucher et al* (1964), *Helmer & Judson* (1963), *Pickens et al* (1965) and *Vito & Fasciolo* 1965) With these methods, which demand from 10 to 25 ml of plasma, renin, after total or partial inhibition of the angiotensinase activity, is alowed to react *in vitro* with renin substrate, angiotensinogen, and the angiotensin produced is measured

The present study describes a microtechnique demanding only 300 myl of plasma The total inactivation of angiotensinase is made unnecessary by determination of the reduction of the substrate concentration instead of measuring the angiotensin produced This principle is generally used in enzymology, and *Munoz et al* (1943, 1946) have used it in the determination of renin in human serum

METHODS

The *renin* used was the commercial preparation of NBC Cleveland Ohio The *substrate* used was a plasma pool from albino rats that had been nefrectomized 24 hours earlier pH was adjusted to 3 6 by 2n of phosphoric acid for 20 minutes at 25 degrees Celcius and subsequently to 7 5 by 6n of NaOH After this process with the renin preparation used, and the temperature applied with an incubation time of 10 minutes and with the concentration of angiotensin reached no measurable angiotensinase activity is found (*Bing* 1964) On the other hand angiotensinase activity is found by increasing the time of incubation

1 *The Durability of the Substrate*

Analyses in duplicate containing 100 myl of substrate + 100 myl of trisbuffer pH 7 5 are incubated in siliconized tubes at 37 degrees C for a time varying from zero to 72 hours

The solution is covered by 600 myl of a mixture consisting of equal parts of toluene and paraffin oil Toluene is used as a bacteriostatic paraffin oil is added to avoid evaporation After this incubation 800 myl of a 10 minutes preheated renin solution (trisbuffer pH [illegible] containing 0 1 Goldblatt Dog Unit (G U) per ml is added to half of the tubes in order to determine the concentration of angiotensinogen and 800 myl of trisbuffer pH 7 5 is added to the other half of the tubes in order to detect [illegible] [illegible] of the substrate The mixture is incubated for

This work is s[illegible] [illegible] grants from *Statens Almindelige Videnskabsfond* and *Reinholdt W Jorck og Hustrus Legat*

10 minutes at 37 degrees C *The procedure to follow consists in admixture of 10 myl of 2n HCl* and placing the tube in boiling water for 10 minutes The precipitate is removed by centrifugation and 10 myl of 2 n NaOH is added to the supernate which is then stored at —20 degrees C. The concentration of angiotensin in the supernate *is determined* as described below, and is expressed as nanograms (ng) per ml

2 *Formation of Angiotensin at Varying Substrate Concentrations*

Trisbuffer pH 7 5 is added to an amount of substrate ranging from zero to 500 myl until the total volume is 500 myl After preheating of both solutions this mixture is incubated with 500 myl of a renin solution containing 0 16 G U per ml (in tris buffer pH 7 5) The concentration of renin during the reaction is identical with that used for determination of the durability of the substrate and transforms 30 per cent of the substrate during a 10 minutes' incubation at 37 degrees C The procedure to follow as described above (1) In order to determine the total substrate concentration this scheme is followed by using 100 myl of substrate and a renin solution containing 2 G U per ml

3 *Measurement of Known Renin Concentrations*

A series of 3 identical tubes a b and c is produced each containing 100 myl of the same renin solution (in trisbuffer pH 7 5) and 100 myl of substrate The concentration of the renin solution is varied from 0 0001 G U per ml to 0 0006 G U per [illegible] containing 100 myl of trisbuffer pH 7 5 and [illegible]

37 degrees C Then tubes a and b are inc[illegible]
with 800 myl of a preheated renin solution containing 0 1 G U per ml while tubes c and d are incubated for 10 minutes at 37 degrees C with 800 myl of preheated trisbuffer pH 7 5 The procedure to follow as described above (1)

4 *Measurement of Renin in Plasma*

The rats are sacrificed by [illegible]
heparinized tube which is [illegible]
centrifugation it is submit[illegible]
strate 100 myl of plasma [illegible]
section "Measurement of Known Renin Concentrations"

5 *Biological Assay of Angiotensin*

[illegible] on is measured by its pressor effect in
fe[illegible] igh c 180 g are given a ganglioplegi[illegible]
ag[illegible]
ba[illegible]
2[illegible]
from 1 to 6 ng of angiotensin a[illegible]
tensin are used The standard [illegible]
till they give almost the same pressor response The sample to be assayed is given between two standards on either side and this is repeated not less than three times for each sample From the two standards before and after the unknown sample a linear interpolation is made to the sensitivity at the time of injection of the unknown The difference if any from this value is counted linearly in the calculation *of the concentration*

RESULTS

The Durability of the Substrate

It is a condition of the determination of the renin activity from the reduction of the substrate concentration that the substrate should be

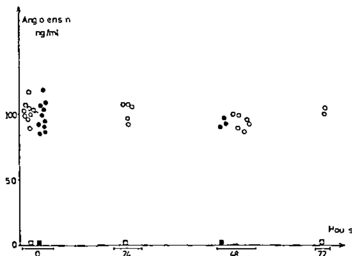


Fig 1

The marks ○ and ● ind cate the durability of two substrate pools after incubation for up to 72 hours at 37 degrees C. The marks □ and ■ indicate that pressor substances are not produced

free from renin and durable in the experimental conditions In order to get a substrate free from renin plasma from rats nefrectomized 24 hours earlier is employed The concentration of angiotensinogen in such plasma is 10 times as high as in normal plasma *(Bing* 1964)

If the substrate is placed as described in Methods 1 from 0 to 72 hours at 37 degrees C the substrate concentration is found not to have changed (Fig 1) In addition it is seen from Fig 1 that by being stored the substrate does not produce any pressor substances Consequently the substrate is free from renin stable and not digested by proteolytic enzymes in the time used (After being stored at —20 degrees C for a long time (3 months) one of the substrates was decomposed during the incubation and an angiotensin like pressor substance was produced In the analysis used a tube d is included to certify that this has not happened)

Angiotensin Produced at Varying Substrate Concentrations

If the concentration of renin is kept constant and the concentration of the substrate is varied as mentioned in Methods concentrations of angiotensin are found after 10 minutes incubation is shown in Fig 2 The concentration of angiotensin is seen to vary in proportion to the concentration of the substrate The concentration of angiotensin produced corresponds to a 30 per cent transformation of the substrate *Bing* (1964) with the same renin and the same sort of substrate preparation has shown that there is the sam propor n in the case of a 20 per cent tr fo mation of the substrate

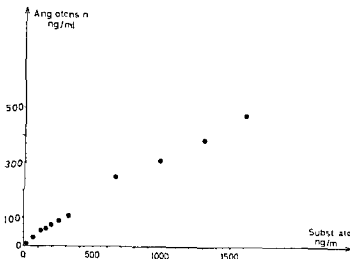


Fig 2

Shows the angiotensin produced at the varying concentrations of substrate The concentration of substrate is expressed in ng of angiotensinogen measured as angiotensin The concentration of renin (0 08 G U/ml) and the time (10 min) are kept constant

Measurement of Known Renin Concentrations

The method includes 3 assays of angiotensin as described in "Methods" The *a value* is an expression of the sum of the concentrations of angiotensinogen from the unknown sample and the substrate at the start of the incubation

The *b-value* is an expression of the remaining concentration of angiotensinogen after incubation for a number of hours, plus the c-value The *c value* is the concentration of angiotensin after incubation for a number of hours, *i e* the part of the angiotensin produced that has not been digested by the angiotensinase

The difference b-c is, consequently, an expression of the concentration of the angiotensinogen remaining after incubation for a number of hours

The concentration of substrate is c 1600 ng of angiotensinogen measured as angiotensin per ml at the start of the reaction From Fig 2 it *is seen that at a constant concentration of renin the angiotensin* produced is proportional to the concentration of substrate at any time When the angiotensin produced corresponds to the reduction of the concentration of the substrate, the concentration of renin in the reaction can be calculated by 1 order kinetics[1]

---

1 From Fig 2 the formation of angiotensin is seen to be proportional to the concentration of the substrate (S) For these low concentrations of renin further *more the angiotensin produced is proportional to the concentration of the enzyme* (Γ) Thus the reduction of the concentration of the substrate per time unit $-\frac{dS}{dt} = k \cdot S \cdot \Gamma$ Integrating over times from t = o to t = t, when Γ is kept constant we get equation (1) (*Moore* 1964)

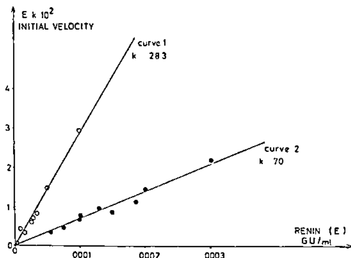


Fig 3

Indicates the initial velocity of the enzyme reaction at low concentrations of renin Curve 1 and 2 have been attained with two different substrate pools, prepared in the same way and with the same concentration of angiotensinogen (This is seen in Fig 1) Marks as in Fig 1 The ordinate is proportional to the initial velocity since the concentration of substrate at the start of the reaction is kept constant

as

$$\frac{2\,303\ \log\left(\frac{a}{b-c}\right)}{t} = E\ k \qquad (1)$$

The left side of the equation can be calculated, t being the time in hours The number 2 303 is the conversion factor from natural to common logarithm By this E k can be calculated, E being the concentration of renin and k a constant

In order to determine k, as mentioned in "Methods" a series of renin solutions with varying concentrations, corresponding to the physiological concentrations, is produced and E k calculated If E k is plotted against the known concentrations of renin (E), the constant (k) is determined as the slope of the curve (Fig 3, curve 1)

The straight line found is assumed to indicate that, within the exactitude of the assay, the reaction is in accordance with 1 order kinetics The straight line is produced with the incubation time varying from 19 to 48 hours, independently of the concentrations of renin This shows that renin is stable during the whole period of incubation Furthermore, the straight line is seen to pass through the zero point of the co-ordinate system, which corresponds to the fact that the whole reduction of the substrate concentration is exclusively due to renin

Measurement of Renin in Plasma

When a standard curve as curve 1 Fig 3 is available, the concentration of renin in plasma can be determined as described in "methods"

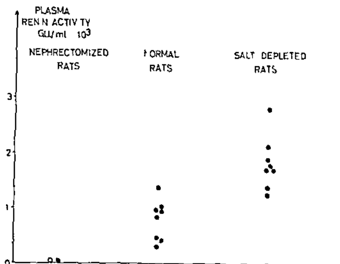


Fig 4

The renin activity in plasma is measured in 8 normal and 8 saltdepleted rats using double tests No renin activity is found in two plasma pools from nefrectomized rats (see Fig 1)

by calculating E k according to equation (1) as described above Inhibitors or accelerators of the renin-angiotensinogen system in the plasma in question are able to modify the result

The method is applied to 8 normal rats and 8 rats which have been saltdepleted through feeding from 4 to 28 days with potatoes and fresh distilled water The results are seen in Fig 4

Factors Modifying the Velocity of the Renin Angiotensinogen Reaction

If the substrate used in Fig 3 curve 1 is incubated with the concentration of renin increased to 0 025 and 0 080 G U per ml, the proportionality is seen to continue (Fig 5) since the slope of the curve is unchanged The formation of angiotensin is measured direct by 10 minutes' incubation In this period, as mentioned above, no demonstrable angiotensinase activity is found In the determination of the velocity of the reaction the total angiotensinogen concentration at the start of the reaction replaces the a-value, and the difference between the total angiotensinogen concentration at the start of the reaction and the concentration of angiotensin produced by 10 minutes' incubation replaces the quantity b c in equation (1)

For another substrate pool, given exactly the same treatment as the substrate pool used in Fig 3 curve 1, we get another velocity of the reaction when low concentrations of renin are applied (Fig 3, curve 2) For higher concentrations of renin we get the same velocity of the reaction in the two substrate pools (Fig 5) This seems to indicate that in the substrate used for curve 2, Fig 3 there is an inhibitor which makes itself felt only in low concentrations of renin

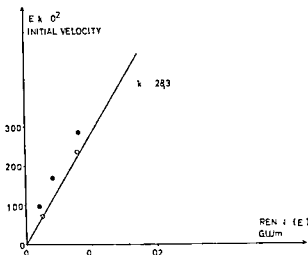


Fig 5

Indicates the initial velocity of the enzyme reaction at higher concentrations of renin The two curves have now within the exactitude of the determinations the same slope corresponding to that of curve 1 Fig 3 Marks as in Fig 3 The determination for 0 08 G U /ml is the average of 8 determinations for each pool Comparison with Fig 3 indicates the presence of a renin inhibitor only of consequence to low concentrations of renin

DISCUSSION

It is desirable to measure renin under conditions similar to those in whole plasma, without inactivating angiotensinase In the method described this demand is partly satisfied, the activity of angiotensinase being only slightly inactivated by a transitory acidification, which renin is known to tolerate without loss of enzymatic activity (*Haas et al* 1963)

Total inactivation of angiotensinase activity is made unnecessary since the principle of the analysis is determination of the reduction of the substrate concentration instead of measurement of the angiotensin produced The determination of the concentration of the substrate is easily made in a plasma with an angiotensinase activity so high that the determination of the physiological concentrations of renin in plasma by measuring the angiotensin produced would be impossible

In addition to the angiotensinase activity the concentration of the substrate is controlled during the incubation since the variation in the concentration of angiotensin gen in the plasma in question cannot influence the result because this variable is included in the determination of the reduction of the substrate concentration Th velocity of the reaction would then be expected to be proportional to the concentration of renin in plasma

However the difference between curve 1 and Fig 3 seems to indicate the presence of an unknown fa tor in plasma f om nefrecto

mized rats (and probably also in that of normal rats) which alters the velocity of the renin-angiotensinogen system

This unknown factor is different from renin, angiotensinogen, and angiotensinase, and is measured independently of the increase in the concentration of angiotensinogen developed after nefrectomy (*Bing* 1964) For high renin concentrations no difference is seen between the two plasma pools, but for low *renin concentrations the velocity* is decreased in one of the plasma pools This was to be expected for a renin inhibitor *Boucher et al* (1964) and *Pickens et al* (1965) have observed that the formation of angiotensin is more rapid in some human plasma than in others

By determination of the renin activity in plasma *from normal and* saltdepleted rats with the method described, the renin activity is found to be highest for the saltdepleted animals This is in accordance with the results obtained by *Brown et al* (1963) and *Genest et al* (1966) for human plasma and by *Fasciolo et al* (1964) for dog plasma

SUMMARY

A simple microtechnique for quantitative measurement of renin in plasma is described For each determination 300 myl of plasma is used The lowest concentration of renin measured is *0 0001 Goldblatt* Dog Unit per ml, but the sensitivity limit can be increased by increasing the time of incubation The renin activity is found to be higher in saltdepleted than in normal rats, while no activity is found in 24 hours nefrectomized rats Variability in the velocity of the reaction found in two pools of plasma from nefrectomized rats may be explained by the presence of an unknown factor, probably a renin inhibitor, in the plasma

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aminocaproic acid (EACA) It should therefore be possible with such a therapy after trauma to accentuate the coagulation disturbance and thus increase the tendency to thrombus formation

The purpose of this study was after intravenous injection of tumour cells to investigate in isologous tumour host systems I The effect of trauma on the number, average volume and distribution of metastases in two different systems II In one of the systems to investigate the effect of heparin and EACA and also the combinations of trauma and EACA on the same factors

MATERIAL AND METHODS

The experimental studies have been performed on inbred C3H mice with a spontaneoues mammary cancer and on CBA mice with a 20 methylcholantrene induced rhabdomyosarcoma MCG1 (*Hellgren et al* 1966)

A The C3H mice originated from Jax Mice Laboratory USA 1962 The mammary cancer developed in a female mouse 1963 and has thereafter been transplanted intramuscularly This study was performed on the 22nd generation Spontaneous

were counted in a hemacytometer and the viability of the cells was checked by nt of

were

Group I	20 mice given 0 1 ml saline intravenously 3 hours before the tumour cell injection
Group II	20 mice given 2 mg heparin (Vitrum Stockholm) in 0 1 ml solution subcutaneously two hours before and 3 hours after the tumour cell injection
Group III	20 mice given 30 mg EACA (Kabi Stockholm) in 0 1 ml solution intravenously two hours before and 1 and 4 hours after the tumour cells injection
Group IV	20 mice given 0 1 ml saline intravenously 2 hours before the tumour cell injection One hour before the cell injection both thighs were crushed with pliers under ether anesthesia fracturing the bone and crushing the muscles
Group V	20 mice treated as in Group III and also subjected to crush fractures as in Group IV

The effect of heparin on the coagulation time and of EACA on the inhibition of fibrinolytic activity were tested as described previously (*Boeryd* 1965) The coagulaton time was not below 15 *minutes during 1*- *hours after the first heparin injection* The time for lysis of the coagulum in the EACA treated mice did not fall [illegible] f two hours before and 7 5 hours after the

k s BSS were njected in a tail vein The e in Group II and 11 mice in group IV died

B 30 CBA mice were devided into two groups

Group I	10 mice without treatment
Group II	20 mice subjected to bilateral crush fracture of the thighs one hour before the tumour cell injection

The tumour cell suspension of MCG1 was prepared according to *Madden & Burk* (1961) with some modifications by *Boeryd et al* (1965). Cell viability was tested with Trypan blue and was in the suspension used 96 per cent. The cells were counted in a hemacytometer.

50 000 cells in 0.1 ml modified Parker 199 (*Sall et al* 1954) were injected in a tail vein. The observation period was 18 days. 3 mice in group II died during this period and were discarded.

In both experiments the lungs and livers were examined microscopically. The number, average and total volumes of metastases were estimated as described previously (*Boeryd et al* 1966). Differences between groups were tested by Wilcoxon's two sample rank test (*Hyrenius* 1962). Difference with $P < 0.05$ were accepted as significant.

RESULTS

A. *C3H mice.* At autopsy gross metastases were found only in the lungs. Microscopical examination of the lungs and livers disclosed metastases only in the lungs of all animals. The results are given in Table 1.

TABLE 1

Metastases to Lungs in C3H Mice

Groups		Incidence of takes	N	v 10^{6}	V 10^{3}
I	Controls	20/20	2293	11	24
II	Heparin	18/18	3003	9	28
III	EACA	20/20	4868	17	84
IV	Trauma	9/9	4792	8	39
V	Trauma + EACA	20/20	9264	14	134

Heparin treatment increased the number of metastases to the lungs and reduced their average volume, but their total volume was unchanged compared to the controls.

EACA treatment increased the number, and average and total volumes of metastases to the lungs compared to the controls.

Trauma increased the number and total volume of metastases but left the average volume unchanged compared to the controls.

When trauma was combined with EACA the number as well as the total volume of metastases to the lungs were increased, but the average volume was not changed compared to the controls.

When the EACA-treated group was compared with the traumatized EACA-treated animals, the number of metastases and their total volume were found to have increased in the latter group, the average metastasis volume tended to be reduced ($0.1 > P > 0.05$).

When the traumatized mice were compared to the traumatized EACA-treated mice, an increase of the number and total volume of metastases was evident in the latter group but there was only a tendency to increased average volume ($0.1 > P > 0.05$).

B. *CBA mice.* At autopsy gross metastases were found in the liver

and in 4 of 10 controls and 6 of 17 traumatized animals in subcutaneous tissue, kidneys, mesentery, diaphragm, pelvic fat tissue and lymph nodes. The gross metastases did not differ significantly between the two groups. Microscopical examination disclosed that 9 of 10 controls and all traumatized animals had metastases to the lungs, all controls and 15 of 17 traumatized animals to the liver. The results are given in Table 2.

TABLE 2
Metastases to Lungs and Liver in CBA Mice

	Incidence of takes	N	$v \times 10^{-6}$	$V \times 10^{3}$
		To lungs		
Controls	9/10	127	16	2
Trauma	17/17	516	50	26
		To liver		
Controls	10/10	116	840	97
Trauma	15/17	58	748	43

Trauma increased the number, average volume and total volume of metastases to the lungs but reduced the number of metastases to the liver. The average volume of metastases to the liver was unchanged but the total volume tended to diminish ($0.1 > P > 0.05$).

DISCUSSION

Previous studies on the effects of heparin and EACA on the metastasizability of intravenously injected MCG1 tumour cells (*Boeryd*, 1965, 1966) demonstrated that thrombus formation around the tumour cells was neither necessary for, nor seemed to promote, the establishment of metastases in that tumour host system. For prolonged heparin treatment merely redistributed the metastases from the lungs to the liver, although the hepatic metastases were larger than in the controls.

In the present investigation the number and average and total volumes of pulmonary metastases from intravenously injected MCG1 tumour cells were increased after trauma, whilst the number of metastases to the liver was reduced. Obviously this must have been due to tumour cell retention in the lungs. Trauma is attended by both increased tendency to thrombus formation and disturbed microcirculation. But as thrombus formation around the tumour cells seems not to promote metastasization in this system, the disturbed microcirculation may have been the causative factor modifying metastasis development in the lungs. Tumour cell retention could also be due to increased aggregation of tumour cells which may lead to increased average tumour volume.

The C3H mice had no gross extrapulmonary metastases but this does not exclude the presence of minute such metastases elsewhere

Heparin treatment was in these animals followed by an increase of the number of metastases to the lungs a result opposite to that observed by other authors using homologous systems (*Clifton & Agostino* 1962 1963 *Wood* 1961 1964) but agreeing with results obtained by *Boeryd* (1966) using an isologous ascites tumour in CBA mice The mammary cancer in C3H mice and the ascites tumour are similar in that they produced metastases almost solely to the lungs after intravenous tumour cell injection The increased number of metastases after heparin treatment could be due to heparin facilitating transcapillary passage and repeated recirculation of tumour cells (cf *Koile* 1964) in time causing a higher number of cells to be trapped in the lungs This in turn could be due to that heparin reduces trapping of tumour cells in thrombi The results also could be due to that heparin reduces aggregation of tumour cells In this tumour host system thrombus formation aroud tumour cells is not a pre requisite for the establishment of a metastasis

The effect of EACA on the formation of pulmonary metastases may be due to increased retention of tumour cells in the lung The increased average volume may be due to increased aggregation of tumour cells *or/and inhibition of the spontaneous fibrinolysis exerted by EACA* The formation of microthrombi around trapped tumour cells could thus promote the growth of metastases This promoting effect of fibrin may be due to fibrin being a suitable substrate for the growth of tumour cells (*O Meara* 1958)

The effect of trauma on the number of pulmonary metastases may also be due to increased retention of tumour cells which could result from increased adhesion of tumour cells to the vascular endothelium possibly because of increased thrombus formation or disturbed microcirculation Trauma had however no effect on the average metastasis volume During the first 24 hours after trauma fibrinolytic activity of rat blood is increased (*Rudenstam & Nilsson*) and this could decrease the size of any thrombi formed around tumour cells A diminished fibrin layer around trapped tumour cells may as suggested also interfere with the nourishment and growth of tumour cells and account for the non increased average metastasis volume

When traumatized animals were treated with EACA in order to inhibit the increased fibrinolytic activity thus further increasing the tendency to thrombus formation the number of metastases to the lungs increased markedly The reason for this is probably a more pronounced retention of tumour cells for the same reasons as after only trauma and after only EACA treatment The average volume was unchanged compared with controls tended to increase compared with the traumatized animals (Group IV) but tended to be reduced compared with EACA treated mice (Group III) This change of the average

metastasis volume in comparison with traumatized animals supports the hypothesis that thrombi around tumour cells may promote metastasis growth in this tumour-host system Heparin treatment however, disclosed that such thrombus formation is not a pre requisite for metastasis growth

SUMMARY

The effect of trauma on metastasis formation from intravenously injected tumour cells has been studied in two isologous systems In one of these systems the effects of heparin, EACA and trauma + EACA have been studied too

In one system, CBA mice inoculated with MCG1, trauma increased the number, average and total volumes of metastases to the lungs and decreased the number to the liver

In the other system, C3H mice inoculated with mammary cancer, in which only pulmonary metastases developed trauma increased the number and total volume of metastases, while EACA increased the number, average and total volumes compared to the controls EACA treatment of traumatized animals led to a further increase of the number and total volume of metastases compared to the controls In the latter system, however, heparin treatment also increased the number of metastases to the lungs

These somewhat different effects of trauma in the two systems and the interplay between effects of heparin EACA, trauma, and trauma + EACA have been discussed

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Ph^1 PREVALENCE, PERIPHERAL BLOOD PICTURE AND CYTOSTATIC THERAPY

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Received 15.vii.66

Tough et al. in 1962 reported that the decreasing leukocyte count in the peripheral blood during radiation treatment of the spleen in patients with chronic myelogenous leukaemia (CML) is accompanied by decreasing Ph^1 prevalence in the corresponding blood cultures. Other investigations have since demonstrated that comparable conditions occur during treatment with cytostatics (*Fitzgerald et al.* 1963, *Carbone et al.* 1963, *Whang et al.* 1963). These results, together with demonstration of correlation between frequency of immature myeloid cells in the peripheral blood and Ph^1 prevalence in the corresponding blood cultures (*Fitzgerald et al.* 1963, *Wolf et al.* 1966), suggest that the Ph^1-positive mitoses in the blood cultures originate from the myeloid cell series. Whereas the Ph^1 prevalence in the blood cultures from patient to patient and at different times in the same patient vary just as much as the prevalence of immature myeloid cells in the blood, the frequency of Ph^1 positive cells in uncultured bone marrow is found, in practically all cases, to be 100 per cent or nearly 100 per cent, irrespective of antileukaemic treatment (*Sandberg et al.* 1962, *Fitzgerald et al.* 1963, *Tough et al.* 1963, *Carbone et al.* 1963, *Whang et al.* 1963, *Tjio et al.* 1966).

These investigations deal with essential conditions for the question whether cytogenetic observations can contribute towards illustration of the mechanisms concerned in progression of chronic myelogenous leukaemia. The present work was undertaken with the object of supplementing them with further investigations of the Ph^1 prevalences in the blood during cytostatic therapy and of the relationship between the frequencies of immature cells in peripheral blood and Ph^1 positive cells in blood and bone marrow.

MATERIAL AND METHODS

The cytogenetic materials or[illegible] from 30 pa[illegible]nts with [illegible] haemato logically typical CML. Ph^1 wa[illegible]und in blood [illegible]ure a[illegible] in all of

Supported by a Scholarship from the *Lady* [illegible]

the patients The extent of the blood culture material, the procedure employed in collecting the specimens and of blood culture and preparation of the specimens from the cultured materials is mentioned elsewhere (*Pedersen 1966 a*) where similarly, the principles for selection of the mitoses for cytogenetic analysis and the procedure employed in this analysis are described All of the cultures in the present material were grown with phytohaemagglutinin

The relationship between cytostatic therapy and Ph¹ prevalence in the blood culture was investigated in nine patients who were newly diagnosed and still untreated at the first investigation and in three patients who had been submitted to treatment for 6, 14 and 18 days, respectively, at the first investigation The untreated patients were re examined 41-222 days (average 81 days) later while the three patients who had undergone brief therapy were re examined 44 52 and 62 days (average 53 days) later

The blood samples from which the cell cultures in the material originated were allowed to sediment spontaneously prior to incubation The author undertook differential counting in the supernatant fluid prior to culture corresponding to 45 blood cultures from 23 patients In each case the differential count comprised 400 cells These counts were correlated with the Ph¹ prevalence of the corresponding cultures in order to illustrate further the relationship between the Ph¹ positive and immature myeloid cells

The principles for collecting bone marrow specimens are mentioned elsewhere (*Pedersen 1966 b*) Eighteen bone marrow specimens comprising 550 cells were prepared without preliminary culture according to a modification of *Tjio & Whang's* method (1962) Spreading of the mitoses was undertaken by evaporation of the fixative during air drying In 13 bone marrow specimens comprising 322 cells the fixative was replaced by 60 per cent acetic acid Spreading of the mitoses was subsequently undertaken by evaporation of the acetic acid

The Ph¹ prevalences were calculated in the following manner (No Ph¹ positive cells × 100)/(No Ph¹ positive + Ph¹ negative cells) All cells lacking one or more small acrocentric chromosomes and where Ph¹ could not be identified were classified as Ph¹? cells and were therefore excluded from the formula recorded above

RESULTS

The relationship between cytostatic treatment and Ph¹ prevalence in the blood cultures was investigated by comparison of the frequency of Ph¹-positive cells in nine patients before and after periods of treatment of varying length (Table 1) A corresponding comparison was undertaken in three patients who had been treated with cytostatics for 14 6 and 18 days, respectively, at the time of the first investigation and for 58 68 and 69 days, respectively, at the time of the second investigation (Table 2) In 11 out of the 12 patients the Ph¹ prevalence was significantly lower at the second investigation In one patient (Case 9) the leukocyte count was not available and in another case (Case 1) the differential count at the second investigation was missing In the remaining nine patients, the haematological conditions had improved considerably during the period in which the fall in the Ph¹ prevalence had developed Further, Case 9 showed a considerably improved differential count and Case 4 decimation of the leukocyte count In one patient (Case 8) the frequency of Ph¹-positive cells had certainly diminished but not significantly Corresponding to this the leukocyte count and blood picture were practically unchanged

This parallel development between Ph¹ prevalence and the frequency of immature myeloid cells suggests that Ph¹-positive and myeloid cells

TABLE 1

Haematological Data an I Ph¹ Distribution in Corresponding Blood Cultures Before and After Various Periods of Cytostatic Treatment

Case no	Date of sampling	Days in treatment	Ph¹ positive cells	Ph¹-negative cells	P	Leucocyte count (x10³)	Differential bl(%)*	pmy(%)§	Count my(%)†
1	23 1 63	–	26	7	< 0 0005	332 0	61	2	3
	20 2 63	27	–	47		0 9	8	2	–
	1 7 63	–	43	9	< 0 0005	251 0	4	–	26
	14 8 63	39	13	31		75 4	3	–	13
3	21 8 63	–	23	19	< 0 0005	361 6	9	–	20
	28 11 63	55	1	43		5 3		–	9
4	23 9 63	–	31	18	< 0 0005	190 0	22	–	22
	14 11 63	52	–	47		18 0			
5	4 10 63		18	29	0 0005 0 0010	71 1	3	8	12
	2 12 63	33	2	43		5 8	–	–	–
	15 11 63		41	7	0 0005 0 0010	255 2	1	6	23
	20 1 64	66	24	23		86 6	–	7	10
	9 12 63	–	23	21	< 0 0005	83 2	–	4	18
	23 3 64	81		27		6 4	–	–	1
8	5 2 64		8	21	0 05 0 10	160 0		13	23
	4 6 64	114	13	85		176 0	4	–	28
9	23 3 64	–	9	39	0 001 0 005	190 4	1	16	30
	31 10 64	17		50			–	–	8

* myel blasts
§ pr myelocytes
† myelocytes

TABLE 2

Haematological Data and Ph¹ Distribution in Corresponding Blood Cultures After Different Periods of Cytostatic Treatment

Case no	Date of sampling	Days in treatment	Ph¹ positive cells	Ph¹ negative cells	P	Leucocyte count (×10³)	Differential bl(%)*	Differential pmy(%)§	Count my(%)†
10	13 7 63	14	46	2	< 0 0005	104 0	4	4	19
	26 8 63	58	21	22		45 6	–	–	16
11	29 11 63	6	37	10	< 0 0005	205 0	–	3	14
	20 1 64	68	2	45		11 0	–	–	3
12	11 1 64	17	36	12	0 005	186 0	–	10	24
	3 3 64	69	21	26		36 2	–	4	9

* See Table 1

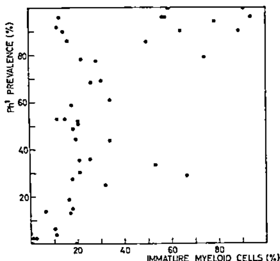


Fig 1

Percentages of immature myeloid cells in 45 leukocyte suspensions and Ph^1 frequencies in blood cultures established from the leukocyte suspensions

are identical. An attempt has been made to illustrate this question further in Fig 1 where the results of the differential counts are compared with the Ph^1 prevalences in the corresponding blood cultures The differential counts were carried out on the cell suspensions from which the blood cultures were established Fig 1 shows the frequency of immature myeloid cells (myeloblasts, promyelocytes and myelocytes) in 45 cell suspensions from 23 patients Suspension investigations were not carried out in four patients Two patients showed peculiar conditions and are, therefore, not included in Fig 1 but will be dealt with separately later

It is apparent from Fig 1 that there is a certain connection between the proportions of immature myeloid cells in the suspensions and the proportions of Ph^1-positive cells in the corresponding blood cultures The points in the diagram, however, show great scatter Possible causes of this scatter will be discussed later The relationship between the prevalences of immature and Ph^1-positive cells describes an S-shaped curve The proportions of Ph^1-positive cells show variations particularly in suspensions with 10–30 per cent immature myeloid cells As it must be presumed that lymphocytes are the precursors of the Ph^1 negative cells in the cultures (*Carstairs* 1962, *MacKinney et al* 1962, *Porter & Cooper* 1962, *Elves & Wilkinson* 1963), and as the lymphocyte prevalences of the suspensions are not taken into consideration in drawing the diagram, it may be conceived that varying lymphocyte counts are responsible for the scatter of the Ph^1 prevalences In 23 cultures, 10–30 per cent of immature cells were present In Table 3, these cultures are subdivided into three groups according to the Ph^1 prevalences As is

apparent, the lymphocyte content of the suspensions diminishes with increasing Ph[1] frequency

Table 3 suggests that the proportion of Ph[1] positive cells in the blood cultures is determined by the balance between the content of lymphocytes and granulocyte precursors in the initial leukocyte suspension In order to illustrate this relationship in more detail the cultures from Fig 1 were subdivided in Table 4 according to the content of lymphocytes and granulocyte precursors in the initial leukocyte suspension As is apparent from the Table leukocyte suspensions with more or less uniform proportions of lymphocytes show higher Ph[1] prevalences the higher their content of granulocyte precursors Suspensions with more or less uniform content of immature myeloid cells appear to result in Ph[1] prevalence inversely proportional to the initial proportion of lymphocytes

TABLE 3

Average Lymphocyte Percentages in Blood Cultures with Different Ph[1] Frequencies and 10 30 Per cent Myeloid Cells

Ph[1] frequency (%)	No of cultures	Average lymphocyte frequency (%)
0 40	9	6 4
41 60	7	4 4
61 +	7	2 1

TABLE 4

Frequencies of Immature Myeloid Cells and Lymphocytes in 45 Leukocyte Suspensions and Ph[1] Prevalences in Corresponding Blood Cultures

Lymphocytes (%)		Immature myeloid cells (%) 0 19	20 29	> 29	Total
0–4	Cultures	7	4	11	22
	Ph[1] positive cells	155	101	361	617
	Ph[1] negative cells	161	74	77	312
	Ph[1] prevalence (%)	49 1	57 7	82 4	66 4
> 4	Cultures	13	5	5	23
	Ph[1] positive cells	153	116	115	384
	Ph[1] negative cells	462	120	104	686
	Ph[1] prevalence (%)	24 9	49 2	52 5	35 9

As mentioned previously peculiar relationships were found between the content of granulocyte precursors in the blood and the content of Ph[1] positive cells in two patients One of the patients (Table 5) a male aged 43 years was in a state of clinical and haematological exacerbation during the period of investigation from the time of the second investigation on 12 10 1962 Despite intensive treatment with cytostatics and corticosteroids no noteworthy improvement took place in his

TABLE 5

Peripheral Blood Picture and Ph¹ Distribution in Blood Cultures and Direct Bone Marrow Specimens from a Patient in Acute Exacerbation of Chronic Myelogenous Leukaemia

Date of sampling	Tissue	Ph¹-positive cells	Ph¹-negative cells	Ph¹ prevalence(%)	Leucocyte count (×10³)	Differential count (%) bl*	pmy*	my*
14.8.62	marrow	36	–	100.0	45.0	–	1	31
12.10.62	marrow	16	–	100.0				
	blood culture	4	38	9.5	62.4	58	5	3
15.10.62	blood culture	1	12	7.7	16.0	28		8
17.10.62	blood culture	1	48	2.0	20.4			
17.1.63	blood culture	4	41	8.9	16.2	36	–	2
31.1.63	blood culture	–	21	–	168.0	90	–	2
5.2.63	blood culture	–	20	–	291.0	98	–	–
11.2.63	blood culture	1	32	3.3	248.0	90	–	–

* See Table 1

TABLE 6

Blood Picture and Ph1 Distribution in Blood Cultures and Direct Bone Marrow Specimens from a Patient Before and After Acute Transformation of Chronic Myelogenous Leukaemia

Date of sampling	Tissue	Ph1 positive cells	Ph1 negative cells	Ph1 prevalence(%)	Leucocyte count (x10³)	Differential count (%) bl*	pmy*	my*
23 9 63	marrow	17	–	100 0				
	blood culture	31	18	63 3	190 0	22	–	22
14 11 63	blood culture		47		18 0			
16 4 64	marrow	21		100 0				
	blood culture	6	49	10 9	20 4	65	5	5
23 4 64	blood culture	1	49	2 0	13 0	40	2	8
24 6 64	blood culture	5	99	4 8	13 6	33	4	3

* See Table 1

condition and this patient died on 12 2 1963 from bronchopneumonia In connection with the two first investigations a bone marrow biopsy showed 100 per cent Ph^1 prevalence Bone marrow investigations could not be carried out later on account of thrombopenia with a haemorrhagic tendency Despite the immature blood picture the Ph^1 prevalences were always found to be under 10 per cent The karyotypes of the Ph^1 positive cells examined did not show any abnormalities apart from sporadic hypodiploidy

The second patient (Table 6) a female aged 46 years (identical with Case 4 in Table 1) was investigated for the first time prior to the commencement of treatment with cytostatics On this and a later occasion Ph^1 frequencies in the bone marrow of 100 per cent were found The first blood culture showed a high prevalence of Ph^1 positive cells At the next investigation the patient was in a state of good remission The content of Ph^1 positive cells in the blood culture was in good agreement with this viz 0 per cent At the subsequent investigations the condition was clinically and haematologically poor but the Ph^1 frequencies in blood culture were low Scarcely half of the Ph^1 positive marrow and blood culture cells from April and June 1964 had the same aneuploid karyotype with 47 chromosomes the (21 22) group containing two Ph^1 This patient died from bilateral bronchopneumonia six weeks after the last cytogenetic investigation

The prevalence of Ph^1 positive cells in uncultured bone marrow was investigated in 31 biopsy specimens from 24 patients and comprised a total of 872 analysed cells Of these 792 were Ph^1 positive 28 Ph^1 negative and 52 cells proved impossible to classify

Twenty seven of the 28 Ph^1 negative bone marrow cells originated from one particular sample of bone marrow the cells of which were distributed in the following manner Ph^1 positive 6 Ph^1 negative 27 and Ph^1? 9 The sample originated from a male aged 70 years whose illness commenced in 1961 with fatigue night sweats loss of weight and enlargement of the spleen The bone marrow and the peripheral blood showed histological changes typical of the disease Good remission was obtained with Busulfan At the time of the investigation the leukocyte count was 80 000 and was increasing The spleen could be palpated 10–12 cm below the left costal margin Unfortunately no differential counts from the blood or bone marrow are available from this period The patient did not exhibit any clinical or haematological signs of bone marrow insufficiency at any time Culture of a blood sample withdrawn simultaneously with the marrow sample was undertaken but no growth resulted As the patient died from cerebral haemorrhage (18 10 1962) [illegible]ek after the cytogenetic investigation this was not repeated

Where seven of the bo[illegible] marrow spec[illegible]mens wer[illegible] concerned no differential counts from t[illegible] ripheral [illegible] [illegible]lable The remaining 24 samples are [illegible] d [illegible] in Table 7 on

the basis of the frequency of immature myeloid cells in the blood Regardless of the character of the peripheral blood picture, the prevalence of Ph¹-positive cells in the bone-marrow was 100 per cent or practically 100 per cent

TABLE 7

Percentages of Immature Myeloid Cells in Peripheral Blood and Ph¹ Prevalences in 24 Direct Bone-Marrow Specimens

Blood picture Immature myeloid cells(%)	Samples	Ph¹ positive cells		Ph¹ negative cells		Ph¹? cells
		Number	prevalence(%)	Number	prevalence(%)	number
0–19	5	134	100 0		–	6
20 39	12	365	99 7	1	0 3	24
> 39	7	154	100 0	–	–	4
Total	24	653	99 8	1	0 2	34

In 18 out of the 31 samples in the bone-marrow material, the mitoses were spread by evaporation of the fixative This material comprises 550 cells of which 52 (9 5 per cent) were hypodiploid In 13 samples, comprising 322 cells of which 163 were hypodiploid (50 6 per cent), the fixative had been replaced by 60 per cent acetic acid and spreading of the mitoses was undertaken by evaporation of the acetic acid The proportion of hypodiploid cells was significantly higher in the latter group ($P < 0\ 0005$), probably because the high temperature necessary to evaporate the acetic acid resulted in rupture of the cell membranes As the high hypodiploid frequency appears to have been artificially conditioned, the material treated with acetic acid was not included in the bone-marrow material which was published previously (*Pedersen* 1966 *b*)

DISCUSSION

In 1962, *Tough et al* described a parallel decrease in the leukocyte count and the Ph¹ prevalences in the corresponding blood cultures in six patients with CML during radiation of the spleen Later investigations have demonstrated a corresponding development during treatment with cytostatics (*Fitzgerald et al* 1963 *Whang et al* 1963, *Speed & Lawler* 1964) *Fitzgerald et al* (1963) found, in addition positive correlation between the prevalences of immature myeloid cells in the peripheral blood and Ph¹-positive cells in the corresponding blood cultures Similar observations were made by *Whang et al* (1963) and *Frei et al* (1964)

The results of the present investigation are in good agreement with the investigations mentioned The relationship between the content of myeloid cells in the initial cell suspensions of the blood cultures and their final content of Ph¹-positive cells is dominated, however, by the

great scatter of the observations In addition there does not appear to be proportionality between the two variables as the observations do not describe a straight line but rather an S shaped curve (Fig 1)

As emphasized by *Court Brown & Tough* (1963) numerous factors condition variations in the relationship between the content in the peripheral blood of myeloid cells and the content of Ph^1 mitoses in the corresponding cultures when harvested

The results of differential counting are associated with uncertainty on account of the relatively limited number of cells counted In order to reduce this uncertainty to a reasonable extent each count in Fig 1 was based on 400 cells On the other hand the Ph^1 prevalences in the majority of cultures are based upon 50 cells analysed and thus these values have not inconsiderable uncertainty

Separation of the red and white blood cells prior to establishing of the cultures was undertaken by spontaneous sedimentation of the erythrocytes without addition of phytohaemagglutinin During this sedimentation the cellular composition of the leukocyte population may be altered Comparison of differential counts on whole blood and the corresponding leukocyte suspensions does not suggest any systematic difference In 23 differential counts on whole blood in the present material an average of 30 5 per cent immature myeloid cells was found while the corresponding leukocyte suspensions showed an average of 29 1 per cent In occasional cases however considerable differences between the corresponding counts were observed In order to avoid these sources of error the observations in Fig 1 were based upon counts of the leukocyte suspensions

Incubation periods of different lengths may influence the proportions of Ph^1 positive cells in the culture (*Nowell & Hungerford* 1961) An attempt was made to avoid this source of error by keeping the period of culture as constant as possible in length All of the cultures were harvested after 46 51 hours of culture

Uncontrollable variations in the experimental conditions may be anticipated to influence the ability of the leukaemic cells to grow *in vitro* Further it is possible that the distribution on myeloblasts promyelocytes and myelocytes and the occurrence of abnormal karyotypes in the leukaemic cell population may influence the competition *in vitro* between the leukaemic and non leukaemic cells and thus the Ph^1 frequency in the cultures

The modifying factors mentioned thus appear to be able to explain the scatter of the points in Fig 1 The S shaped distribution of the points is presumable conditioned by variations in the lymphocyte frequencies in suspensions with 10 30 per cent immature myeloid cells (Table 3)

In two patients the Ph^1 prevalences in the blood cultures were found to be very low despite the high frequencies of immature cells in the peripheral blood (Tables 5 and 6) Bone marrow investigations in both

of these patients showed 100 per cent prevalence of Ph^1-positive cells. The number of blood cultures excludes technical factors as the cause of the low prevalences in both patients. Special defects in the myeloid cells concerned appear to have rendered *in vitro* proliferation impossible. *Tough et al* (1961) described two similar cases. Although the clinical course was typical of CML, it is difficult to exclude the possibility that the cases described by *Tough et al* might belong to the rare group of Ph^1-negative patients as bone marrow investigations were not available.

In contrast to the varying Ph^1 prevalences in the peripheral blood, the majority of investigations show constant frequencies of Ph^1-positive cells in uncultured bone-marrow regardless of treatment and phase of the disease. *Tough et al* (1963) thus found only one Ph^1-negative out of 1343 bone-marrow mitoses investigated from 25 patients. However, two patients had already been excluded from this material with exclusively Ph^1-negative bone-marrow and three patients with partially Ph^1-negative bone marrow and clinical and haematological signs of bone-marrow insufficiency. The results of the present investigation are in good agreement with this as all of the Ph^1-negative cells with one exception originate from one patient. This patient, who had 18 per cent Ph^1-positive cells in the bone-marrow did not show any clinical or haematological signs of bone-marrow insufficiency at any time during the period of disease.

Following exclusion of cells with questionable Ph^1, the material published by *Sandberg et al* (1962), which originates from 14 clinically typical patients, contains 9.2 per cent Ph^1-negative mitoses. In 15 samples from 13 patients, *Whang et al* (1963) found 88–100 per cent, on an average of 97.8 per cent Ph^1-positive cells. *Carbone et al* (1963) found 96–100 per cent Ph^1-positive cells in 22 patients. In 14 bone-marrow samples from ten patients *Fitzgerald et al* (1963) demonstrated 24–100 per cent of Ph^1-positive cells, on an average of 73.0 per cent. *Tjio et al* (1966) in their material from 60 patients found that an average of 1.2 per cent of the cells investigated were Ph^1-negative.

These results are possibly only apparently controversial. In the four last mentioned results, differentiation was undertaken only between Ph^1-positive and non-positive cells. The latter group probably includes the mitoses which do not prove possible to classify as regards Ph^1. In calculation of the Ph^1 prevalences the Ph^1-positive cells are compared with the total number of cells investigated which implies that the Ph$^{1?}$ cells are included in the calculation as Ph^1-negative. The genuine frequency of Ph^1-positive cells, *i.e.* the Ph^1-positive fraction of the cells whose content or absence of Ph^1 can be determined with certainty is therefore probably higher than the number stated. In addition, the authors who give the number of Ph$^{1?}$ cells do not define this category more exactly. Hypodiploid cells which lack (21–22 members should be considered as Ph$^{1?}$ cells as artificial factors may

have caused loss of Ph[1] in these cells *Fitzgerald et al* (1963) found very low Ph[1] prevalences in two patients These were apparently patients with both Ph[1]-positive and Ph[1] negative populations in the bone-marrow similar to the three described by *Tough et al* (1963), the patient mentioned in the present investigation and a case described by *Speed & Lawler* (1964) Following exclusion of these two patients, the Ph[1] prevalence becomes 88 3 per cent in the material presented by *Fitzgerald et al* This material contains rather a high proportion of hypodiploid cells i e 21 8 per cent compared with 9 5 per cent in the fraction of the present material which was treated according to *Tjio & Whang's* method (1962), which may be due to artificial chromosome loss Presuming this, it appears possible that the frequency of hypodiploid cells which lack (21–22) members and are included as Ph[1]-negative cells in the calculations of Ph[1] prevalences is quite high and can explain the discrepancy to the other materials published

The investigations quoted thus suggest that the marrow population in the majority of patients with CML consists exclusively of Ph[1] positive cells This finding forms the basis of the presumption that not only the myelopoietic but also the erythropoietic and megakaryocytic systems are Ph[1]-positive (*Sandberg et al* 1962, *Tough et al* 1963) In 1963, *Whang et al* succeeded in demonstrating that the erythrocyte precursors may be Ph[1] positive and that it is probable that they are in many cases *Tough et al* (1963) demonstrated in one case parallelism between the thrombocyte count in the blood and the frequency of polyploid Ph[1] positive mitoses in corresponding uncultured bone marrow samples This finding supports the hypothesis about Ph[1] in the megakaryocytic system The existence of patients with both Ph[1]-positive and Ph[1] negative bone marrow populations may possibly indicate that Ph[1] may occur only in myeloid cells in certain cases (*Whang et al* 1963)

The persistence of 100 per cent Ph[1] prevalence despite intensive antileukaemic treatment suggests that the preleukaemic Ph[1]-negative population becomes totally supressed by Ph[1] positive cells or is no longer capable of proliferation As the dosage of the antileukaemic therapy is, as a rule, regulated according to the effect upon the peripheral blood picture, it seems obvious why the Ph[1] prevalence in the bone marrow does not reflect any therapeutic effect

However, provided other factors are equal, when a Ph[1] positive bone-marrow population exists in an environment containing a cytostatic, the variants whose proliferation is least inhibited by the cytostatic are favourized It is therefore possible that conventional antileukaemic therapy, which has no or only a slight effect upon the bone marrow population, will encourage proliferation and further development of resistant cell variants and consequently development of intractable myeloblastic crises

SUMMARY

In a material of blood cultures originating from 29 patients with clinically and haematologically typical chronic myelogenous leukaemia, the following questions were illustrated. The effect of cytostatic therapy on the Ph[1] prevalence in blood culture and the relationship between the content of lymphocytes and immature myeloid cells in the initial leukocyte suspensions and the corresponding Ph[1] prevalences after culture *in vitro* for 46–51 hours. Finally, the frequency of Ph[1]-positive cells was investigated in uncultured bone-marrow from 22 patients.

1. In 11 out of 12 newly-diagnosed patients of whom nine were untreated at the first investigation and three had received treatment for 6, 17 and 24 days, respectively, marked decrease in the Ph[1] prevalences of the cultures were observed after periods of treatment of varying lengths. Simultaneously, the peripheral blood pictures improved considerably. In one patient, the frequency of Ph[1]-positive cells did not decrease significantly and the blood picture did not show any definite improvement (Tables 1 and 2).

2. Prior to incubation, differential counts were undertaken on 45 leukocyte suspensions from 23 patients for comparison with the Ph[1] frequencies in the corresponding blood cultures (Fig. 1). The observations revealed a greater proportion of Ph[1]-positive cells in the cultures harvested the greater the proportion of immature myeloid cells in the corresponding initial suspensions. The relationship between the two variables is, however, not linear but describes an approximately S-shaped curve. The shape of the curve appears to be caused by varying lymphocytic content in the suspensions. In two patients, low Ph[1] prevalences were found despite very immature peripheral blood pictures. In these two cases, special defects appear to have rendered proliferation of the myeloid cells *in vitro* difficult (Tables 5 and 6).

3. In one patient, only six out of 33 bone-marrow mitoses investigated were definitely Ph[1]-positive. This patient did not differ clinically and haematologically from the other 21 patients from whom bone-marrow investigations are available. These investigations comprise a total of 787 cells which could be classified with certainty as regards the presence or absence of Ph[1]. Of these 787 cells, only one was Ph[1]-negative.

The results of the present investigations are in good agreement with previous investigations and together with these they suggest that the immature myeloid cells of the peripheral blood are identical with the Ph[1]-positive cells of the blood cultures and that decreasing Ph[1] prevalences in the blood cultures during successful therapy are a reflection of the improvement of the peripheral blood picture.

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CYCLES OF HAIR GROWTH IN HAIRLESS MICE

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Received 27 vi 66

A moderate single surface application of a carcinogen to mouse skin gives rise to many tumours when applied during the resting stage of *the hair cycle* whereas few or no tumours are seen when the carcinogen is applied during the growth phase This hair cycle effect has been extensively discussed by *Engelbreth Holm* and his school (see *Borum* 1960)

It has several times been claimed that the hair cycle effect is probably of no or of minor importance in hairless mice *David* (1932) has shown however that hairless mice have rudimentary hair cycles even if these are not easily observed In the study of carcinogenesis it is of importance to know the time of appearance of these cycles We have therefore studied the more or less rudimentary hair cycles in our strain of hairless mice

MATERIAL AND METHODS

Hairless mice of the strain hr/hr were used (*For a more detailed description see Iversen & Evensen* 1962) The animals were housed in plastic cages and fed a standard diet Groups of 4 male and 4 female mice were killed by neck fracture at different time intervals starting at an age of fifteen days (see Graph 1) The skin of each animal was flayed off stretched on a piece of cardboard and fixed in 4 per cent neutral formaline Specimens were taken from the central as well as from the dorsal side and in each case from the cranial the middle and the caudal part of the body The specimens were embedded in paraffin and cut and stained with eosin and haematoxylin The *microscopical staging of hair growth phases* follows the conventional nomenclature (see for instance Argyris 1963)

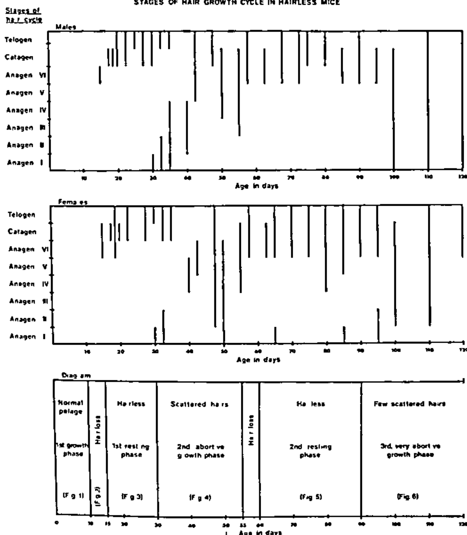
RESULTS

The results are summarised in Graph 1

Hair Growth

Generally no difference between males and females was observed The two sexes are therefore considered together

At birth these mice are hairless but just afterwards they acquire a normal thick first pelage (Fig 1) which they start losing again at the



Graph 1

Stages of hair cycles in hairless mice The vertical bars in the two upper parts represent stages of hair cycle observed in one or more of the animals studied at that age The lower part of the graph is a diagram summarizing the findings, and with references to the photographs of mice at different ages

age of 10 to 12 days The hairs are always lost from the head tailwards (Fig 2), and the loss is complete after about ten days

In the 13-day-old mice many hairs are still fully grown out, but the hair follicles show signs of degeneration (catagen stage, Fig 7) Some few hair bulbs are still well preserved, with normal hairs Mitotic figures are not seen in the hair follicles at this time, and the inter-



Fig 1
A 1 week old mouse with apparently normal pelage

follicular epithelium is thin Signs of degeneration of the hair bulbs are mostly seen in the specimens from the cranial part Two to three days later, however, hair bulbs in the catagen stage are also observed in the remaining part of the skin area

On the 20th day the animals are macroscopically hairless (Fig 3) Microscopically all hairs are in the catagen or in the telogen stage (Fig 8) This condition continues for about ten days

About the 30th day almost all the animals reveal a number of mitotic figures in many hair follicles and during the period from 30 to 40 days, the histological picture is characterized by many hair bulbs in different steps of the anagen stage

At about the 40th day some slight regeneration of hair is macroscopically seen in most animals (Fig 4) This pelage, however, is far



Fig 2
A 13-day old mouse in the hair losing phase

from being so thick as the first one In most of the mice only a few scattered hairs are observed but a small number of animals have a thin fur Other animals again are almost hairless

After the 50th day this second rudimentary pelage is lost and micro scopically almost all follicles are again in the catagen or telogen stage This second resting phase lasts from day 50 until the animals are about 90 days old (Fig 5) Some very few scattered follicles still show signs of hair growth even during this resting phase

On the 90th day a third growth cyclus starts but with only a few active follicles One observes only a few hairs in some of the animals (Fig 6) but many remain hairless Microscopically a few scattered follicles show signs of hair growth



Fig 5
A 70-day old mouse with hairless skin

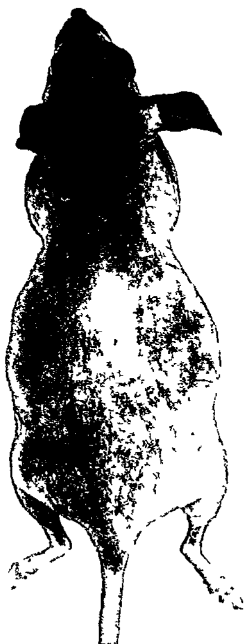


Fig 6

A 100-day old mouse with some hairs. This is an example of maximum hair growth at this age

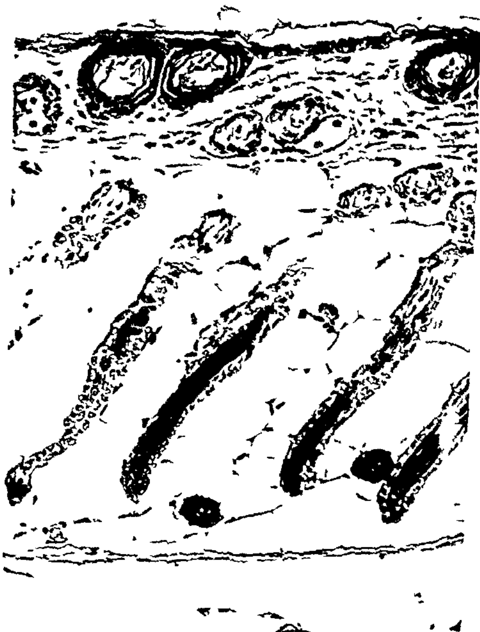


Fig 7

Microscopical section from the skin of a 15 day old mouse with hair bulbs partly well preserved partly with degenerative changes Note the thickness of the skin Initial X — 64



Fig 8
Microscopical section from the skin of a 27 day old mouse Thin dermis without hairs Inactive hair bulbs Initial X=64

to the skin surface (Fig 10) These hairs may be surrounded by an inflammatory infiltrate, which also contains multinucleated foreign body giant cells These cysts and the inflammatory changes remain during the rest of the life of the animals

DISCUSSION

The strain of hairless mice *hr/hr* (sometimes called "Crew mice") is not absolutely devoid of hair Our strain is obtained from P Hertwig Biologisches Institut, Halle, Germany, in 1939, and has since been bred in our Institute The hairless mothers cannot take care of their offspring because their mammary glands are rudimentary In this laboratory *females with hairs heterozygote for hairlessness, are mated to* homozygote hairless males

Female offspring with hairs are used as mothers for the next generation, whereas hairless females are used for experiments The hairless males are used for experiments and for mating but strict brother sister breeding cannot be performed Yet, with this type of breeding during 27 years this strain is if not purely inbred rather homogenetic

Our investigations show that there are at least two distinct growth cycles of the hair bulbs of these animals The first stage of growth



Fig 9

Microscopical section from the skin of a 43 day old mouse Histiocytes and lymphocytes around active hair follicle. Cysts in the corium Initial X — 61

lasts from birth, and the pelage is complete at the age of 10 days The second growth phase starts about day 30, and lasts for about three weeks A third, abortive growth phase starts in some animals 90 days of age

As it is of importance to pay attention to the "hair cycle effect" in the study of carcinogenesis when using single application of carcinogens, such studies with hairless mice of the strain *hr/hr* should preferably start when the animals are in a sufficiently stable resting stage, *i e* between 60 and 90 days of age

SUMMARY AND CONCLUSIONS

Hair growth cycles in the strain of hairless mice (*hr/hr*) are studied Rather distinct phases of growth and rest are observed

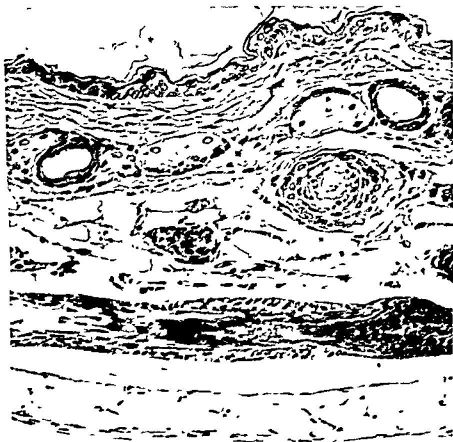


Fig 10

From the same mouse as Fig 9 A hair growing horizontally close to the musculus panniculus Initial X — 64

The first growth phase lasts from birth until the mice are about 10 days old This pelage is apparently normal From the 12th to the 15th day these hairs are lost from the head tailwards.

The first resting phase lasts from the 15th to the 30th day Almost no hairs are observed, and the hair bulbs are in the catagen or in the telogen stage

The second growth phase lasts from the 30th to the 55th day Some few scattered hairs are observed on the skin, and a few of the hair follicles are in the anagen stage

The second resting phase lasts from about the 60th day to about the 90th day of age During this period only very few scattered hairs are seen in some of the mice Microscopically the hair bulbs are almost all in telogen or catagen

After the second resting phase a very abortive third growth phase occurs in some few mice

Inflammatory changes are present from the time of the degeneration of the first pelage *i e* from the 25th day Formations of cysts begin in the second growth phase

For single application of carcinogens during a resting phase mice at an age of 60 to 90 days should be used

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ON THE APPEARANCE OF THYROGLOBULIN AUTO-ANTIBODIES AFTER EXPOSURE OF NORMAL HUMAN THYROIDS TO SURGERY AND IONIZING RADIATION

By

TORE GODAL and PETER BERDAL

Received 12 vii 66

A variety of patients without thyroid disease may be exposed to thyroid injuries Tracheostomy frequently requires splitting of the thyroid isthmus and malignant tumors of the neck may be treated by x rays striking the thyroid, or by surgery including intervention on the gland Whether these injuries may initiate thyroid auto-immune reactions, appears to remain unknown Investigations of such patients may both throw light upon this question and upon the mechanism by which thyroglobulin auto antibodies reach detectable levels in human serum

At present, possible mechanisms for the induction of thyroglobulin auto-antibodies may be summarized as follows

1 The "leakage theory" (4 13) By this hypothesis thyroglobulin is assumed to be secluded from the immune system in the tolerance developing period If thyroglobulin later gets entry to the circulation the molecule is recognized as "foreign" and stimulates antibody synthesis

2 Disturbed tolerance theories These hypotheses assume that tolerance towards thyroglobulin normally develops but may be terminated by either of the following mechanisms
   A An alteration of the thyroglobulin molecule
      1 A structural change such as denaturation presenting "hidden" antigenic determinants (17)
      2 Addition of new antigenic determinants (28) Viruses may give rise to such new antigenic determinants of cells (26) or cellular products (23)
   B Secondary to thyroid disease not involving structural changes of thyroglobulin Lymphoid cells may be attracted to the thyroid gland for other reasons than auto immune reactions By an infiltration of this kind, inter actions between thyroglobulin and lymphoid cells different from the fate of thyroglobulin in lymph nodes may take place and favour auto antibody synthesis
   C Hyperactivity of the immune system A disturbance located to the immune system "spontaneously giving rise to so-called "forbidden clones" (3)
   D Molecules of the external mil eu such as ant gens of micro organisms may have antigenic de erm nants closely related to tissue c mponents and give rise to cross reacting antibo lies (1

Since human thyroglobulin auto-antibodies frequently are found in a variety of thyroid disorders (24) many of which most unlikely have an auto immune etiology, the auto-antibody production seems to be secondary to the thyroid disorder Furthermore, in many of these thyroid disorders the immune system reveals no sign of hyperactivity (9) On this basis, hypotheses 2 C and D may be excluded as main mechanisms behind thyroglobulin auto antibody formation

Several observations do not support the "leakage" theory, as discussed by *Hjort* (13) One of the arguments against this theory is the lack of antibody response in patients after surgical treatment of thyroid disease, although surgical intervention on the thyroid gives rise to circulating thyroglobulin (10) Since circulating thyroglobulin frequently is found in serum of patients with thyroid disorders (11), the explanation could be that the selected group of patients without antibodies had become tolerant to thyroglobulin previous to surgery Consequently, auto-antibody response after leakage of thyroglobulin should be studied in patients without thyroid disease

The present report concerns thyroglobulin auto-antibody response in patients without clinical evidence of thyroid disease after injuries of their thyroid gland

MATERIALS AND METHODS

The material consisted of 99 patients from the Ear Nose and Throat Department at Rikshospitalet Oslo 86 patients suffered from tumors located to larynx or upper part of trachea These patients were treated either by laryngectomy including hemi thyroidectomy or by external x rays which strike the thyroid The total x ray dosis varied from 2000 R to 4000 R to one area on each side of the neck corresponding to a tissue dose in the thyroid of approximately 3400–6200 R One patient suffered from a cerebral tumor and another from cervical spondylitis Both were tracheostomized with splitting of the thyroid isthmus The 11 last patients suffered from tumors of their larynx These are included in the control group because serum specimens were only achieved prior to treatment

TABLE 1

Time Interval between Thyroid Injury and Serum Sampling

Type of injury	Surgery	x rays
No of patients tested ≦ 3 months after injury	11	25
" 4–6 "	3	13
" 7 12 " "	6	16
" 2 5 years "	10	28
" ≧ 6 "	7	11

One or more serum samples were obtained from each patient at varying periods of time after treatment as shown in Table 1 Twenty five patients were tested both before and after treatment The interval between treatment and antibody testing of these patients varied from 2 weeks to 6 months

Thyroglobulin antibody was demonstrated by the indirect hemagglutination technique as previously described (6 7 8) employing formalinized human erythrocytes containing approximately 35 000 antigen molecules per cell (8) The erythro

cytes were used in a concentration of about 4 × 10 cells per ml counted by photometry (6). Sensitivity was controlled by a positive reference serum obtained from the Department of Bacteriology and Immunology, University of Buffalo School of Medicine, Buffalo, N.Y. (Head: Professor E. Witebsky, M.D.). The sera were treated as previously described (7) and tested in dilutions of 1:5, 1:25, 1:250 and 1:2500 made by a single pipette. Sera with a positive titer = 1:2500 were tested further in ten fold dilutions for hemagglutination activity and undiluted in gel precipitation.

Precipitation in gel *ad modum Ouchterlony* (19) was performed as described by *Eriksen* (5) employing 0.03 M barbital buffer at pH 8.6, 0.003 per cent methyl orange, 1 per cent Difco Special Agar Noble and 0.1 per cent phenol.

RESULTS

The results are presented in Table 2. Out of 54 patients exposed to x rays only, 3 patients (5.6 per cent) revealed thyroglobulin auto antibodies in their serum. Two of these were tested 3 years after treatment. One patient, a 50 years old male, revealed a titer of 1:25 while the other, a 60 years old male, gave rise to a titer of 1:250. The third patient was a male of 74 years who both 6 weeks and 1 year after treatment showed an antibody titer of 1:25 000 and a positive gel precipitation reaction. The last case is described in more detail below (Case 1).

TABLE 2

Age and Sex Distribution of Patients without Thyroid Disease Exposed to Different Types of Thyroid Injury

Age in years		21–30		31–40		41–50		51–60		61–70		71–80		81–90	
Sex		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Type of injury	Total no. tested														
x rays	54	1		1		4	1	16	3	15	2	8	1	2	
No. positive	3					1		1				1			
Surgery	14				1			5		6		1	1		
No. positive	1									1					
Surgery and x rays	7				1			3		2		1			
No. positive	1									1					
x rays and surgery	13				1	1		3		6	1	1			
No. positive	0														
Tested prior to injury	36				2	3	2	7	3	9	1	7		2	
No. positive	1									1					

Of 14 patients exposed only to surgery 1 patient, a 67 years old male operated 7 years prior to serum sampling, gave a positive reaction (1:25). Twenty patients were exposed both to x rays and surgery. In a single patient thyroglobulin antibodies were detectable. Serum obtained 6 weeks after a biopsy of the thyroid and 2 weeks



Fig 1

Thyroid tissue from patient F T Arrow points to the infiltration of a squamous cell carcinoma surrounded by lymphocytes and fibrous tissue In peripheral parts thyroid follicles with scattered lymphocytic aggregation may be seen (Hematoxylin and eosin × 90)

after the beginning of x-ray treatment to the neck revealed a titer of 1 2500 and a negative gel precipitation reaction This patient was tested 6 months prior to any treatment with a negative result and is described in further detail below (Case 2)

Altogether 5 out of 88 patients (5 7 per cent) with injured thyroids revealed thyroglobulin antibodies, while 1 out of 36 patients tested prior to any treatment gave a positive reaction In 1 out of 25 patients a negative reaction previous to treatment was followed by a positive afterwards (Case 2)

Case 1 A male (J B) born 1891 was admitted to the hospital on September 4th 1964 for treatment of a right vocal chord carcinoma No history of previous thyroid *disease and no signs of struma or hypothyreosis by physical examination* at admission

From September 8th to October 20th he received a total amount of 3850 R to each side of his neck Shortly afterwards he began to feel sensitivity to cold dryness of the skin tiredness and he became constipated

He was admitted for control of his laryngeal carcinoma on December 9th 1964 His tumor had then regressed However his face was swollen and he appeared suspect of myxoedema His thyroid was not palpable Investigations Protein Bound Iodine (PBI) 0 9 μg per ml cholesterol 380 mg per 100 ml I^{131} uptake 4 per cent/4 hours and 3 per cent/24 hours Thyroglobulin antibodies hemagglutination titer 1 25 000 gel precipitation Positive

Treatment with I thyroxine 0 10 mg × 3 was *initiated and his symptoms of hypo* thyreosis disappeared gradually Serum specimens from this patient obtained 4 6 and 12 months after x ray treatment gave identical results A hemagglutination titer of 1 25 000 and a positive precipitation in gel

Case 2 A male (E T) born 1900 was admitted to the hospital on May 10th 1965 because of hoarseness for the last few months Four months earlier he obtained an x ray examination because of dysphagia and a Zenker s diverticulum was dia gnosed He was found to have a left sided paralysis of the recurrent nerve His thyroid was not palpable Bronchoscopic and oesophagoscopic examinations did not reveal any pathological changes except for the diverticulum of hypopharynx

He was discharged from the hospital and controlled one month later and on October 4 The first time he was without symptoms except for the persistence of the recurrent paralysis but the last time he complained about cough and respiratory distress At that time and in addition to the recurrent paralysis a supraclavicular lymph node on the left side suspect of metastasis was observed For this reason he was readmitted to the hospital

The respiratory distress was by bronchoscopy found to be caused by a tumor in the upper part of the trachea Tracheostomy was performed on October 15 Tumor *infiltration was found on the tracheal wall and in the upper part of the thyroid* Histology showed a squamous cell carcinoma originating in the trachea The stroma around the tumor revealed a dense inflammatory reaction with infiltration of lymphoid cells and granulocytes Also the metastasis to the thyroid apparently had attracted lymphoid cells as shown in a picture of the thyroid (Fig 1)

On October 25 x ray treatment of an 8 × 10 cm area below the jugulum was

May 14 1965	Indirect hemagglutination negative
November 29 1965	Indirect hemagglutination 1 2500 Precipitation in gel negative
March 16 1966	Indirect hemagglutination 1 2500 Precipitation in gel negative

DISCUSSION

Thyroglobulin auto antibodies were found in the serum of 3 patients out of 54 (approximately 5 6 per cent) without clinical signs of thyroid disease exposed to x rays of their thyroid in 2 out of 34 patients (5 9 per cent) exposed to thyroid surgery or surgery and x rays and in 1 out of 36 patients (2 8 per cent) tested prior to thyroid injury These relative frequencies are not significantly different and the frequencies 5 6 or 5 9 per cent are not higher than the incidence of thyroglobulin auto antibodies in ordinary patients without thyroid disease which in Europe varies from 5 per cent (24) to 11 per cent (12) In only 1 out of 25 patients a negative reaction previous to treat *ment was followed by* a positive after treatment Since this patient (Case 2) had a metastatic infiltration of his thyroid from a tracheal carcinoma the appearance of thyroglobulin auto-antibodies may have been unrelated to surgery or x rays Consequently the danger of auto antibody formation against thyroglobulin by the described types of surgical and radiologic injury s to normal human thyroids seems to be very small

One of the patients (Case 1) developed hypothyreosis following x ray treatment of a laryngeal carcinoma At the same time a high

5

titer of thyroglobulin auto-antibodies (1 : 25 000) was found. Although patients with post-irradiation myxoedema more frequently contain thyroglobulin auto-antibodies than postirradiation euthyreotic patients (2), the cause of this coincidence remains obscure. The explanation may be that such patients prior to treatment already had a *latent or unapparent autoimmune reaction towards their thyroid* which was enhanced by radiation and provoked myxoedema (18). This hypothesis may also explain why ionizing radiation on thyrotoxic glands (18) in contrast to radiation on normal thyroid glands, as shown by the present study, frequently initiates thyroglobulin auto-antibody production.

Hjort (10) has shown that thyroglobulin gains entrance to the circulation after surgical injuries of the thyroid gland. *Robbins* (21) and *Robbins et al.* (22) could detect circulating thyroglobulin after administration of therapeutic dosis of I^{131}. *Lindsay et al.* (15) observed pathological changes in thyroids consisting of follicular atrophy and fibrosis in patients treated with x-rays for laryngeal cancers. One of the present patients developed myxoedema subsequent to x-ray treatment of a laryngeal cancer. Consequently, external x-rays employed for treatment of tumors of the laryngeal region do affect the thyroid gland. Furthermore, ionizing radiation as well as surgical damage, apparently injure the thyroid in such a way that thyroglobulin gains entrance to the circulation. But this leakage does not seem to stimulate thyroglobulin auto-antibody synthesis. On this basis the present results represent evidence against the "leakage" theory.

The present results are in agreement with those of *Hjort* (10), who detected thyroglobulin antibody after exposure to thyroid surgery in only 1 of 29 patients with thyroid disease who prior to surgery had not had thyroglobulin auto-antibody in their serum. Furthermore, the "leakage" theory is not supported by experimental investigations. Intravenous injections of thyroglobulin do not elicit antibody formation (25) and a destruction of thyroid structure by I^{131} with an expected leakage of thyroglobulin does not stimulate thyroglobulin auto-antibody synthesis (27).

Consequently, the production of thyroglobulin auto-antibody seems to imply a breakage of tolerance either by an alteration of the antigen itself (hypothesis 2 A) or it may be secondary to infiltration of the thyroid gland by lymphoid cells (hypothesis 2 B). Our Case 2 may support the latter hypothesis. Since x-rays and thyroid surgery do not seem to induce thyroglobulin auto-antibodies, this patient may have started to produce such antibodies as a consequence of the neoplastic invasion of the thyroid. The explanation may be that the lymphoid cells which surrounded the malignant cells in the thyroid have initiated thyroglobulin auto-antibody synthesis after contact with thyroid elements. Since apparently not all kinds of lymphoid cell infiltration of the thyroid do elicit thyroglobulin auto-antibodies (25, 27), some spe

cial interactions between thyroid elements and lymphoid cells may be required

Interaction between lymphoid cells and other cells is a well established phenomenon and is referred to as emperipolesis (14). Emperipolesis has been observed between lymphocytes and tumor cells, lymphocytes and megakaryocytes, lymphocytes and cells undergoing mitosis (14) and between lymphocytes and thyroid cells mainly from Hashimoto strumas (20). The latter interaction has been shown to be a property of the thyroid cells not being specific for lymphocytes from Hashimoto patients (15). Thus emperipolesis seems not to be an *in vitro* demonstration of cell bound immunity. Whether special types of lymphocytes exclusively participate in this reaction seems to remain unknown. Even if phenomena such as emperipolesis may be of importance in breakage of tolerance to autologous thyroid tissue, the role of antigenic alterations in this process or as a single factor remains unanswered.

SUMMARY

Thyroglobulin auto antibodies are rarely detected in patients without thyroid disease whose thyroid has been exposed to x rays and/or surgery. Of 54 patients mainly suffering from laryngeal cancers thyroglobulin auto antibodies were demonstrated in 3 (5.6 per cent) following the administration of a tissue dose of approximately 3400-6200 R of the thyroid gland. Among 34 patients exposed to surgical injury or surgery and x rays of the gland 2 (5.9 per cent) were found to have thyroglobulin auto-antibodies. One patient out of 36 (2.8 per cent) revealed thyroglobulin auto antibodies prior to any treatment. In 1 out of 25 patients a negative reaction previous to treatment was followed by a positive after treatment. This patient was found to have an infiltration of the thyroid from a tracheal squamous cell carcinoma.

Since thyroglobulin apparently gains entrance to the circulation following thyroid injuries of the kinds studied, the results represent evidence against the "leakage" theory of thyroglobulin auto antibody formation.

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Acta path et microbiol scandinav 69, 71 76, 1967

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A METHOD FOR QUANTITATIVE EVALUATION OF THE NUCLEAR DISTRIBUTION IN THE GLOMERULI OF RENAL BIOPSY SPECIMENS

By

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Received 15 vii 66

In 1936, *Bell* (1) described a method for the quantitative evaluation of a socalled "glomerulitis", by which he understood hypercellularity in a glomerulus *Bell* drew all the endothelial and epithelial cells in the glomerular tufts in histological sections of the kidney, and found among other things that the ratio of endothelial cells to epithelial cells in the glomerular tufts was less than one in 84 1 per cent, equal to one in 15 per cent and greater than one in 0 9 per cent, in a material of 107 individuals who had died following an accident The ratio equal to one was described as "glomerulitis" group 0, and ratios greater than one were graduated as "glomerulitis" group 1, 2, 3, etc , dependent on the magnitude of the figure

In the following, a more simple method is described for evaluation of the total number of *cell nuclei* in the glomerular tuft (endothelial and epithelial cells), per unit of area in a histological section of a kidney biopsy specimen

The original reason for the employment of the method was the wish to evaluate the number of cell nuclei in the glomerular tuft in a material of 28 patients with rheumatoid arthritis compared with the number of cell nuclei in a normal control group of 11 patients This study will therefore be briefly described as an example of the utility of the method (*Brun et al* 1965 (3))

MATERIAL AND METHODS

The normal control group comprises renal biopsies from 11 patients (7 women and 4 men) the mean age being 46 years (27 60 years) In these patients a peroperative transperitoneal renal biopsy was performed in connection with operation for gallstone The type of biopsy needle was the same as that used for the percutaneous biopsies (*Iversen & Brun* 1951 (5) and *Brun & Raaschou* 1958 (2)) The patients had normal endogenous creatinine clearance and Addis Shevky's concentration test no proteinuria normal blood pressure and they had not previously had symptoms of diseases of the kidney or urinary tract The histological sections of the

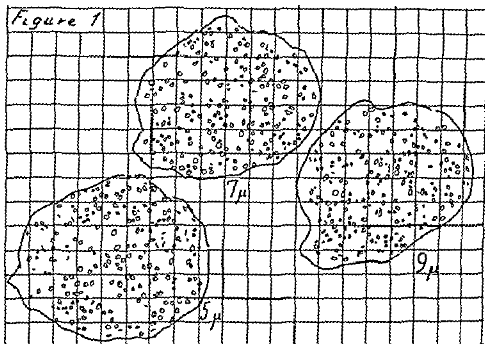


Fig 1

The figure shows 3 neighbouring sections of the same glomerulus in a control biopsy, the individual thickness being 5, 7 and 9 μ respectively, drawn by the technique described in the text. The drawing is divided into standard squares of 625 μ^2

biopsy specimens were described as normal on examination by light microscopy

The kidney tissue obtained was fixed, embedded in paraffin and cut in c. 7 μ sections, subsequently the section thickness was measured optically in the microscope by focussing on lower and upper surfaces (*Pearse* 1961 (4)). The quantitative evaluation was carried out on sections stained with haematoxylin and eosin

The cell nuclei in the glomerular tuft were counted by means of a microscope with a drawing device. The glomeruli were projected on to the paper and a line drawn corresponding to the inside of Bowman's capsule

All cell nuclei in the glomerular tuft were then drawn and counted (Fig. 1). Only the well preserved glomeruli were recorded. The area of the single glomerulus was measured by means of a planimeter and the values for each biopsy were summed up

The significance of variations in thickness of the preparations was examined *with a view to the influence on their mutual comparability*. If the section thickness is equal to or less than one nuclear diameter, more than one whole cell nucleus cannot be present at any one point of the section. Within this limit, therefore, minor variations in section thickness have no significant influence on the number of nuclei in the section. This is illustrated in the following way. Fig. 2 shows one of four drawings consisting of equally large circles of diameter *d* at the same mutual distance *a*, which on the four drawings is equal in turn to 0 d (i.e. the circles touch each other), 0.5 d, 1.0 d and 1.5 d respectively. On each drawing 10 "sections" (parallel lines of constant length) are drawn at random to give a thickness of 0.5 d, 0.7 d, 0.9 d, and 1.1 d. The number of circles intersected by each section is counted, and the mean value and variance calculated. Table 1 shows the significance of the difference in number of circles intersected in sections of different thickness in each drawing. Variations in section thickness within the value of the circle diameter are seen to have no or little significance

In the biopsies the maximum diameter of the nuclei (equatorial sections) were

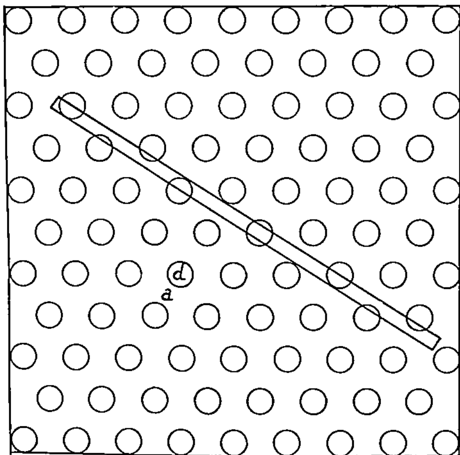


Fig 2

The figure shows uniform circles with a diameter of *d*. The distance (a) between 2 neighbouring circles = *d*. A section of thickness = 0.5 *d* lies across the drawing. See text.

found to be 8–10 μ and the distance between the nuclei varied from zero up to four or five times the diameter of the nucleus.

In order to test the above argument the cell nuclei were counted in three neighbouring sections of the same three randomly chosen glomeruli in a biopsy from one of the controls, the sections being 5, 7 and 9 μ thick, respectively. Table 2 shows the nuclear counts in the three glomeruli in the different section thickness. No significant variation is found in the nuclear counts for the individual glomeruli when the section thickness changes from 5–9 μ.

RESULTS

The count method was applied for two purposes, which will be discussed here as a practical application:

1) to determine the mean number of nuclei per unit area of the glomerular tuft (1000 μ^2), which for the eleven patients was 7.8 ± 1.2

nuclei (mean value ± standard deviation) In the group of 28 patients with rheumatoid arthritis, where kidney biopsy was performed percutaneously, the mean value was 7 0 ± 1 7 in 15 patients without proteinuria and with normal serum creatinine and 7 7 ± 1 5 in 13 patients with elevated serum creatinine and/or proteinuria These values do not differ significantly

TABLE 1

Relationship between Thickness of Section and Number of Circles which are Intersected by the Section on the Drawings

Drawing	Circle diameter	Circle distance (a)	Significance of difference in number of circles intersected in sections of different thickness as compared with a section of thickness 0 5 d		
			0 5/0 7 d significance*	0 5/0 9 d significance*	0 5/1 1 d significance*
1	d	0 d	—	—	+
2	d	0 5 d	—	+	+
3	d	1 0 d	—	—	+
4	d	1 5 d	—	—	+

* — = no significance + = significance ($p < 0.05$) See text

TABLE 2

Number of Cell Nuclei from 3 Randomly Selected Glomeruli in 3 Adjacent Sections of Different Thickness in a Biopsy from the Control Group

Section thickness µ	Glomerulus No 1	Glomerulus No 2	Glomerulus No 3
5	163	212	185
7	153	208	187
9	168	219	197

2) To estimate whether localized accumulations of cell nuclei might be present in the glomeruli in patients with rheumatoid arthritis as it often had been our impression that this might be the case in some glomeruli

To elucidate the problem of a possible localized accumulation of cell nuclei in the glomeruli, the glomerular cell nuclei have been counted in a standard square with a side of 25 µ (Fig 1) This area has been selected since the possible histological element appeared to be of this order of magnitude

By examination of the distribution of the nuclear count per unit area (625 μ^2) it is possible to show whether or not the cell nuclei occur

in localized accumulations If there is a localized accumulation of cell nuclei in the glomeruli the number of squares containing many nuclei will be increased in comparison with the distribution found in the control group Likewise the number of squares with no or few cell nuclei will also be increased since it was found that the total number of nuclei per unit area remained unchanged from the rheumatoid arthritis group to the control group

By means of statistical analysis (probit diagrams), the number of cell nuclei per square of $625\ \mu^2$ were found to be distributed in a uniform pattern in biopsies from the eleven controls In the rheumatoid arthritis group the spread of values obtained in the individual biopsies was slightly greater, and besides the variation in the spread between the individual biopsies was slightly greater than that in the biopsies obtained in the control group

On this basis it was decided to compare the ratio between mean (x) and variance (s^2) for each biopsy ($s^2/\bar{x}$) as the shape of the curve in the probit diagrams recalls the binominal or Poisson distribution Calculation showed that this ratio on an average was about 0 75 for the control biopsies For the biopsies in the rheumatoid arthritis group it was significantly higher (Wilcoxon s rank test) For further references see *Brun et al* (1965 (3))

A localized accumulation of cell nuclei in the glomeruli is seen in patients with rheumatoid arthritis but not in the controls But the phenomenon was observed only in connection with other histological lesions in the kidneys (amyloidosis chronic interstitial nephritis) and was not observed in otherwise normal kidney biopsies from these patients

DISCUSSION AND CONCLUSION

In a given specimen of renal tissue (kidney biopsy or autopsy specimen) it may be very difficult to determine whether it is a matter of diffuse glomerular hypercellularity (diffuse glomerulitis) or of a localized accumulation of cells (localized glomerulitis)

The above mentioned method has proved to be applicable for the solution of a concrete problem Have patients with rheumatoid arthritis diffuse and/or localized hypercellularity in the glomerular tufts in the kidneys in contrast to the controls?

The reason why the method has been described as a simple method is that it is easy to carry out unfortunately however it is troublesome and very time consuming On the other hand the method makes it possible to photograph suitable glomeruli in a given specimen instead of drawing them to count the cell nuclei measure the area of the glomerulus on the photo under standard conditions and compare the result with the average number of cell nuclei per unit area (*e g* $1000\ \mu^2$) in a normal control group of reasonable magnitude

nuclei (mean value ± standard deviation). In the group of 28 patients with rheumatoid arthritis where kidney biopsy was performed percutaneously, the mean value was 7.0 ± 1.7 in 15 patients without proteinuria and with normal serum creatinine and 7.7 ± 1.5 in 13 patients with elevated serum creatinine and/or proteinuria. These values do not differ significantly.

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			0.5/0.7 d significance*	0.5/0.9 d significance	0.5/1.1 d significance
1	d	0 d	—	—	+
2	d	0.5 d	—	+	+
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SUMMARY

This paper provides a description of a method for quantitative evaluation of the number of cell nuclei in the glomerular tufts in kidney biopsy specimens

The application of the method is illustrated by an example An evaluation is made to see whether patients with rheumatoid arthritis have a diffuse and or localized hypercellularity in the glomerular tufts of the kidneys The method has proved to be applicable to this concrete problem but in a modified form it may be possible to apply it generally to selected problems On the other hand the method cannot be recommended as a routine as it is time consuming and troublesome

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Methods a and b seemed to give comparable coating of the rods, whereas method c may be less reliable because droplets of vaseline set on the "edges". Method a or b was used in the majority of experiments carried out

In our experiments, the material sampled from air was transferred to culture medium by pulling the rod over a plate, simultaneously performing zig zag movements If a heavy harvest of microbes is to be expected, it is necessary to pull the rod over 2-3, or even more, consecutive plates in order to obtain a quantitative measure of the air contamination The choice of medium is dependent upon the species to be isolated In our experiments we used tryptose agar (Difco) with 10 per cent blood

TABLE 1

Comparative Sampling with Roto Rod Sampler and Exposed Open Blood Plates

Dates of sampling 1965	Roto-Rod Sampler Colonies sampled per minute, equal 40 litres of air Hours					Exposed open blood plates Mean numbers of colonies per plate per minute exposed Hours				
	07 30	10 00	12 00	15 00	17 00	07 30	10 00	12 00	15 00	17 00
20 Sept	65 2	73.2	45 2	134 0	31 3	6 67	2 00	1 00	6 00	1.33
21 „	216 0	69 6	144 7	61 3	31 6	25 00	1 80	3 00	3 03	0 50
22 „	96 4	29 0	50 5	260 0	34 2	1 48	0 45	1 68	16 41	0 91
27 „	106 4	74 0	164 8	182 2	27 0	6 23	1 88	1.28	2 09	0 77
28 „	334 6	53 4	67 2	102 2	22 6	9 01	1 79	0 75	2 94	0 29
29 „	90 8	112 8	96 6	271 0	78 0	6 63	2 73	1 40	14 93	1 11
4 Oct	73 6	65 6	67 0	500 2	46 8	1 13	3 11	1 05	8 18	0 58
5 „	222 2	110 4	156 0	689 2	100 0	2.30	1 03	1.20	8 00	0 93
6 „	95 0	360 2	151 4	569 2	88 6	1 59	3 63	1 20	10 17	1 17
11 „	399 4	112 4	12 4	132 8	25 2	5 99	0 53	0 23	1 07	0 31
12 „	593 6	68 6	58 8	195 0	33 0	8 67	0 66	0 61	4 57	0 36
13 „	216 0	44 2	13.2	51 8	52 2	2 21	2 83	0 55	0 91	0 77
Sum	2515 7	1173 4	1027 8	3348 9	570 5	77 01	22 44	12.27	78 29	9 03
Mean	209 8	97 8	85 6	279 1	47 5	6 40	1 90	1 00	6 50	0 75
Mean per litre	5 25	2 45	2 14	6 98	1 19					

A pilot series of experiments were carried out in a comparatively large laboratory in the department of bacteriology, with the Roto-Rod Sampler placed on a shelf in the middle of the room, 1 60 m above floor level All four "edges" of the rod were coated with vaseline, and the apparatus was run 10 minutes clockwise, and immediatly switched over and run 10 minutes counter clock-wise The harvest was very poor, only a few colonies of micrococci and B subtilis were found, a result which have been rather confusing if we had not as a controlmeasure exposed plates of medium at the same time and the same place Having completed the pilot study in the laboratory, the Roto Rod Sampler was tested in various departments of the hospital Particular attention was paid to the handling of used bed linen, which was checked step by step from bed changing until it was placed in the washing machine Variable amounts of microbes were sampled by the rod, and pathogenic [illegible]

at 15⁰⁰ just after the beds were sm thed cut after visiting time and the final fifth sampling was timed at 17⁰⁰ when little or nothing was going n in the room The sampling was carried out over peri ds of three consecutive days in all 4 periods



Fig 1

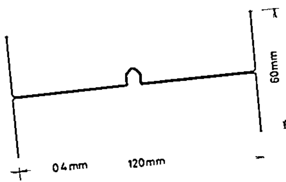


Fig 2
Form and dimensions of the Rod

2 To perform comparative sampling with the Roto Rod Sampler and exp sed open plates of medium

The "edges" of the rod must be coated with a thin layer of some sticky medium For their particular purpose the expert of the mentioned research establishment used silicon grease Being ignorant of the tolerance of microbes to silicon grease we selected sterilized vaseline for the coating of the "edges" of the rods

The rods were coated with vaseline in one or another of the following ways

a Sterile melted vaseline was spread over a flamed knifeblade and the "edges" of the rod were rubbed over the blade
b Instead of a knife blade the vaseline was spread over a sterile glass plate and the rods were processed as mentioned under a
c The edges of the rods were dipped in melted vaseline

Methods a and b seemed to give comparable coating of the rods whereas method c may be less reliable because droplets of vaseline set on the 'edges' Method a or b was used in the majority of experiments carried out

In our experiments the material sampled from air was transferred to culture medium by pulling the rod over a plate simultaneously performing zig zag movements If a heavy harvest of microbes is to be expected, it is necessary to pull the rod over 2 3 or even more, consecutive plates in order to obtain a quantitative measure of the air contamination The choice of medium is dependent upon the species to be isolated In our experiments we used tryptose agar (Difco) with 10 per cent blood

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In a four bed room in the surgical department sampling was car[illegible] hours five times daily The first sampling took place at 7[30] just af[illegible] The second and third samplings were performed at 10[00] and 12[00], [illegible] represents a comparativel q iet time in the ward The f urth [illegible] at 15[00] just after the beds wer smoothed out after visiting time [illegible] sampling was timed at 1[illegible] when little or nothing was going [illegible] sampling was carried ut over periods of three c nsecutive days [illegible]

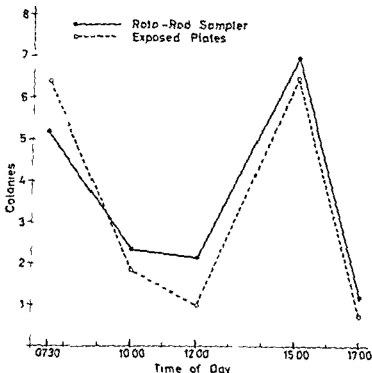


Fig 3

Number of colonies (microbes) sampled with the Roto Rod Sampler from per liter air examined compared with the mean number of colonies (microbes) per minute per exposed open plate

In all experiments *the* Roto Rod Sampler was compared with 10 exposed open *blood* plates *The 3 first days the Roto Rod Sampler* was run for *10 minutes and the* following days for five minutes The material collected on the "*edges*" of the rods were transferred to plates as described above The first five sampling days the plates were exposed for 1/2 hour the following sampling days for 15 minutes

RESULTS

The results of *these experiments are shown* in Table 1 *They confirm* what was already known, that the contamination of the air is a function of activity in the room Regarding the comparative sampling, there are singular days and hours when results obtained with the Roto-Rod Sampled and the exposed plates seem to disagree However, *on the* whole, the average of the results obtained with the Roto-Rod Sampler *and the* exposed plates *compare* very well This is nicely demonstrated in Fig 3 *where the number of colonies* per liter *air sampled* with the Roto-Rod Sampler *are compared with the* mean *number of colonies* per plate per minute exposed

When testing the Roto-Rod Sampler we were faced also with the question how to determine the optimal *length of time* to run the *rod*

TABLE 2

Results of one, two and five Minutes Consecutive Sampling with Roto Rod Sampler

Sampling time No of tests	1 minute Side a	b	2 minutes Side a	b	5 minutes Side a	b
1	17	18	25	12	28	28
2	18	15	16	29	40	23
3	20	18	53	21	40	44
4	27	47	63	24	46	50
5	95	20	39	14	27	48
6	171	51	10	109	26	25
7	13	12	51	49	155	135
8	8	11	23	17	34	59
9	7	1	7	19	37	29
10	27	9	24	25	47	41
	403	202	311	319	480	482
Sum a + b	*605*		*630*		*962*	

TABLE 3

Phage Types of 64 Strains of Staphylococcus aureus

Number	Groups	Types
11	III	83 A
11		77Ad B5 D
10	M	82 47 +
2	II	3A/3B
2	M	80 KS6 82 6/7 47 53 54 70/73 77 +
1	M	82 42E 47 54/70 73 77
1	M	82 53 +
1	M	82 83A
1	I	29 52/79 80 KS6
1	I	52
1	IV	42D
22		NT

It was not unlikely that the high speed of the rod might interfere with the vaseline coating on the "edges" in such a way that the ability to take in microbes might deteriorate

A preliminary study of this problem is presented in Table 2

Three rods were run clock wise in consecutive order, the first for one minute, the second for two minutes, and the third for five minutes The two "edges" coated with vaseline were marked a and b, and the material was transferred to blood plates as described above

The results of 10 experiments show considerable variation in the number of microbes sampled with "edges" *a* and *b* in the individual experiments Further, the results seem to indicate that the number of

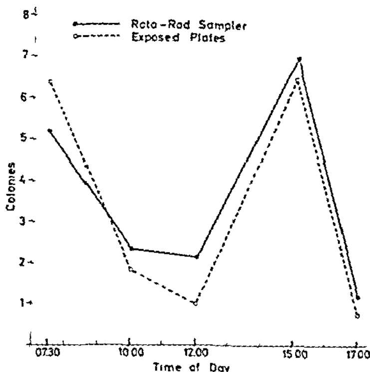


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Acta path. et microbiol. scandinav. 69, 83–91, 1967

The Institute of Clinical Bacteriology (Head: Prof. Sten Winblad, M.D.)
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STUDIES ON *YERSINIA ENTEROCOLITICA*

Characterization of 28 Strains from Human and Animal Sources

By

BIRGITTA NILEHN

Received 3.xi.66

Investigations of biochemical, physiological, and serological properties of the species *Yersinia enterocolitica* (Y. e.), have been published by several authors (*Hässig et al.* 1949, *Dickinson & Mocquot* 1961, *Becht* 1962, *Daniels & Goudzwaard* 1963, *Daniels* 1963, *Akkermans & Terpstra* 1963, *Knapp & Thal* 1963, *Siegmann* 1963, *Mollaret & Chevalier* 1964, *Mollaret & Lucas* 1965, *Frederiksen* 1964, *Carlsson et al.* 1964, *Smith & Thal* 1965, *Wauters & Mollaret* 1965, *Winblad et al.* 1966). Five related bacterial strains were published under the name of Bacterium enterocoliticum by *Schleifstein & Coleman* 1939.

However, most of the strains hitherto described have apparently been isolated from animal sources.

Isolation of such strains from man has only occasionally been reported (*Schleifstein & Coleman* 1939, *Hässig et al.* 1949, *Carlsson et al.* 1964, *Mollaret & Chevalier* 1964, *Wauters & Mollaret* 1965, *Winblad et al.* 1966). Judging from preliminary investigations, Y. e. is a fairly common human pathogen (*Winblad, Nilehn & Sternby*).

The purpose of this paper is to present some characteristics of strains recently isolated from human sources. Seventeen of the strains in this study have been isolated from patients, all of whom except one, had had acute abdominal symptoms, occasionally also diarrhoea. Two of the cases have been reported by *Winblad et al.* (1966). Some older strains from various sources were also included in the tests. Most of the tests were carried out at 37° C as well as at 22° C.

MATERIAL AND METHODS

Bacterial strains. Eleven Y. e. strains from different sources and previously described by *Hässig et al.* (1949), *Becht* (1962), *Daniels & Goudzwaard* (1963), *Daniels* (1963), *Knapp & Thal* (1963), *Siegmann* (1963), *Mollaret & Chevalier* (1964), *Frederiksen* (1964), *Carlsson et al.* (1964), *Smith & Thal* (1965).

microbes sampled do not increase proportionally with prolonged running time

By the studies reported no attempt was made to identify every species taken in by the Roto-Rod Sampler However, in all series of experiments, the cultures were searched for pathogenic staphylococci, and the morphology of other microbes were determined The main lot of microbes was cocci—less frequently rods (*B. subtilis, E coli, Achromobacter*).

Strains of *Staph aureus*, coagulase positive, were isolated in all experiments carried out in the wards Of all strains of staphylococci sampled in the four-bed-room, (the material reported in Table 1) 64 were typed by phages The groups and types presented in Table 3, cover a wide spectrum which may indicate inflow from varied sources The one strain of group IV, 42 D, was the only one isolated from material sampled from the hospital during 1965

CONCLUSION

Our conclusion, based on the preliminary experiments carried out with the Roto-Rod Sampler, indicates that this handy apparatus might be of great value in all efforts to investigate and control microbial air contamination

Further detailed studies of particular problems connected with the use of the Roto-Rod Sampler—such as coating of the "edges", optimal running time, and transfer of the material to suitable medium are needed

For testing of sensitivity to antibiotics human blood agar without peptone added LSU agar (*Juhlin & Ericson* 1961)

Tests for identification

Gram staining Smears were made from 24 and 48 hour cultures on human blood agar

Oxidation/fermentation test Unless otherwise stated 0 5 per cent concentrations of respective test substances in liquid base medium were used in the tests

Base medium Peptone 1 per cent Meat extract 0 5 per cent NaCl 0 5 per cent Bromthymol blue in absolute alcohol 0 0024 per cent Final pH 7 5–7 6 Medium without test substance was used as control

The tubes were inoculated from human blood agar cultures (24 hours 37° C) with a platinum loopful of the culture and incubated at 22° C and 37° C respectively

Quantitative determination of the inoculum was not performed repeated test series however always gave identical results The tubes were inspected after 1 2 3 5 10 20 and 30 days' incubation

Test substances l arabinose (Brit Drug Houses Co Ltd Lond 2390350) l rhamnose (Kebo AB Stockholm Sweden S5100) d xylose (F Merck AG Darmstadt Germany 8692) d glucose (Baker Chem Co Phillipsburg N J., 1916) d fructose (Kebo AB S 5543) d galactose (Merck 4061) d mannose (1 per cent) (Pfannstiehl Lab INC 1219 Glen Rock Ave Waukegan Cleveland Ohio) lactose (Baker Chem Co 2248) sucrose (Baker Chem Co 4072) maltose (Merck 5910) d raffinose (Nutritional Biochemicals Corporation Cleveland Ohio 1-1109), d trehalose (hydrate) [illegible]

Grave [illegible]

per cent [illegible]

redisti [illegible]

Corp ration 12341) salizin (Nutrititonal Biochem Corporation S 3771)

Aesculin splitting was tested according to Cowan & Steel 1965 (Aesculin Merck [illegible]

[illegible] ig to *Bülow* [illegible] 5788 5525 [illegible] mical Com [illegible]

[illegible]

Proteose peptone No 3 0065 01 (Difco Lab Detroit Michigan) 2 per cent di potassium phosphate 0 1 per cent sodium thiosulphate 0 00008 per cent agar 1 5 per cent distilled water, pH 6 7 The medium was tubed and stab inoculated Readings after 1 2 3 5 10 20 and 30 days

Decarboxylase reactions according to *Møller* (1955) l ornithine monohydrochloride 12 75D and l arginine monohydrochloride (Nutritional Biochemicals Corporation) l lysine monohydrochlorid CHR (Fluka AG Chemische Fabrik Buchs SG Switzerland) were used as test substances Medium without test substance was included in the tests

Phenylalanine deamination according to *Shaw & Clarke* (1955) (DL phenylalanine CHR 46628 Fluka AG Buchs SG Switzerland) Incubation time 2 days

Citrate utilization according to *Cowan & Steel* (1965) medium modified from *Köster* (1923)

Voges Proskauer (V-P) reaction according to Barritt (1936) slightly modified The method will be described in detail in a future paper

[illegible] media were made from the test tubes [illegible] ants were investigated in a shortened [illegible] Gram stained smears and registration [illegible] slide agglutination and tests for acid formation from lactose d mannit l and sucrose

Motility studied in 0 2 per cent agar in base medium in tubes

Inoculation was done in incubators at 22° C ± 1° C and 37° C ± 0 5° C The temperature was continously registered with a thermograph

Slide agglutination was done with rabbit antisera prepared against O antigen and

Strains[1]	Original no	Source
334	Siegmann 268	Chinchilla
335, 337, 338 339	Daniels 1975 1905 924 931	Chinchilla
336	Hassig	Homo
347, 348	Becht 18 51	Chinchilla
356	Becht 200	Dog
357	Frederiksen P71	Chinchilla
PX	Winblad	Homo

Strains recently isolated from human sources

6498 Isolated from appendix Patient female, 24 years Diagnosis acute terminal *Ileitis Mesenteric lymphadenitis*

4528 Isolated from appendix Patient female 8 years Diagnosis acute terminal Ileitis Mesenteric lymphadenitis

5525 (5787, 5867) Isolated from appendix respectively faeces Patient female 29 years, with symptoms suggesting acute appendicitis

5674 (5722) Isolated from faeces Patient female, 63 years with diarrhoea abdominal pain fever and acute arthritis

5653 *Isolated from appendix Patient male, 24 years Diagnosis* acute terminal ileitis Mesententeric lymphadenitis

5788 (5845, 5846). Isolated from faeces Patient male 51 years with symptoms suggesting acute appendicitis

5944 (6024) Isolated from appendix respectively faeces Patient female 8 years Diagnosis acute terminal ileitis Mesenteric lymphadenitis

6064 (278, 430 503 489) Isolated from appendix respectively faeces Patient female 21 years *Diagnosis mesenteric lymphadenitis*

144 (146 101) Isolated from faeces Patient female 20 years with acute abdominal pain

564 (754 784) Isolated from appendix respectively faeces Patient male 14 years Diagnosis mesenteric lymphadenitis

582 Isolated from faeces Patient female 26 years hospitalized because of high I S R

953 *(1112) Isolated from faeces Patient female 6 years Acute abdominal pain*

1029 Isolated from appendix Patient male 19 years Acute appendicitis

1077 Isolated from faeces Patient male 11 years Acute abdominal pain

1168 Isolated from faeces Patient female 22 years hospitalized because of diarrhoea arthralgia and erythema nodosum

1691 Isolated from appendix Patient female 46 years Operated upon because of left sided ovarian cyst acute abdominal symptoms

1759 *Isolated from appendix Patient male 24 years Symptoms suggesting acute* appendicitis

Figures in brackets denote code numbers of repeated isolates from the same patient

Primary isolation was done at 22° C and in some cases also at 37° C on LSL agar (*Juhlin & Ericson 1961*)

Pure cultures of both primarily isolated strains and of older strains were obtained by repeated subculture *of apparently pure colonies at 37° C on LSL agar and human* blood agar The strains were then primarily tested regarding their biochemical physiological and serological properties and for their sensitivity to antibiotics

The strains were stored on human blood agar at 22° C for longer intervals on human blood agar at +5° C with subculture every 3rd to 4th week

As soon as pure cultures had been obtained after primary isolations the strains were also freeze dried to ensure material in case of contamination or for supplementary *examinations*

Solid Media Used

Human blood agar (with 1 per cent Proteose peptone no 3 Difco Lab Detroit Michigan)

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1 Strains 334–357 were kindly placed at our disposal by Prof *E Thal* The State Veterinary Medical Institute Stockholm

For testing of sensitivity to antibiotics: human blood agar without peptone added LSU agar (*Juhlin & Ericson* 1961)

Tests for identification

Gram staining Smears were made from 24 and 48 hour cultures on human blood agar

Oxidation/fermentation test Unless otherwise stated, 0 5 per cent concentrations of respective test substances in liquid base medium were used in the tests

Base medium: Peptone 1 per cent, Meat extract 0 5 per cent, NaCl 0 5 per cent Bromthymol blue in absolute alcohol 0 0024 per cent Final pH 7 5–7 6 Medium without test substance was used as control

The tubes were inoculated from human blood agar cultures (24 hours, 37° C) with a platinum loopful of the culture and incubated at 22° C and 37° C respectively

Quantitative determination of the inoculum was not performed, repeated test series however, always gave identical results The tubes were inspected after 1, 2 3, 5, 10 20, and 30 days' incubation

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Ge

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G sorbitol, 1

p , glycerol

r Biochem

Corporation 12341), salizin (Nutritional Biochem Corporation S 3771)

Aesculin splitting was tested according to Cowan & Steel 1965 (Aesculin Merck

according to *Bülow*

[illegible]28, 5653, 5788, 5525

[illegible]gma Chemical Com

Catalase reaction (slide method) H_2O_2 3 per cent in distilled water

1 2 3, 5 10 20 and 30 days

in the tests

Phenylalanine deamination according to *Shaw & Clarke* (1955) (DL-phenylalanine CHR A6628 Fluka AG Buchs SG Switzerland) Incubation time 2 days.

Citrate utilization according to *Cowan & Steel* (1965), medium modified from *Koster* (1923)

Voges Proskauer (V P) reaction according to Barritt (1936), slightly modified The method will be described in detail in a future paper

[illegible] id media were made from the test tubes

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[illegible] f Gram stained smears and registration

[illegible] slide agglutination and *tests for acid*

formation from lactose d mannit l and sucrose

Motility studied in 0 2

Incubation was done in

perature was continuously

Side agglutination wa

OH antigen preparations as well as against suspensions of living bacteria of a known Y e strain (PX) according to *Winblad et al* (1966)

Tube agglutination was done with the use of rabbit O antisera against *strain* PX and O antigen preparations of the strains in question according to *Winblad et al* (1966) *As O antigen* suspensions containing about 10^8 bacteria/ml were used in the tests

Tests for sensitivity to antibiotics were performed according to *Ericsson* (1960 1966)

Filter paper discs with antibiotics and data regarding the regression lines were obtained from Bacteriological Department, Karolinska Sjukhuset, Stockhom Sweden

Discs containing the following substances were used Benzyl *Penicillin* (20 IU) *Methicillin* (30 mcg), Ampicillin (20 IU), Streptomycin (50 mcg), Tetracycline (50 mcg), Oxytetracycline (50 mcg) Chloramphenicol (30 mcg), Kanamycin (5 mcg), Erythromycin (50 mcg), Nitrofurantoin (30 mcg) Neomycin (50 mcg), Sulphonamide (Elkosin) (2 4 mcg), Colistin (900 IU) Nalidixic acid (30 mcg), Fucidin (50 mcg) Novobiocin (50 mcg), Oleandomycin (50 mcg)

The bacteria were suspended in saline and seeded on blood agar plates so as to give dense but not confluent growth on the plates Incubation at 37° C over night

The inhibition zones were measured in mm and the sensitivity was calculated according to regression lines for each antibiotic as determined by *Ericsson* (1960, 1966) The sensitivity was graded as follows *I* = "Sensitive", II = 'Fairly sensitive", *III* = Slightly sensitive', and IV = "Resistant'

RESULTS

Morphology

Gram-stained smears from recently isolated Y e cultures plated on human blood agar showed Gram negative rods with small coccoid forms dominating

Diagnostic Tests

Results of oxidation/fermentation tests at 22° C and 37° C Recently isolated human strains attacked the carbohydrates tested in a very uniform way Thus all formed acid rapidly (1–2 days) from d glucose, d-fructose, d mannose, sucrose, d trehalose, sorbitol d-mannitol at both temperatures No acid was formed from l-rhamnose, lactose, d-raffinose, inulin, dulcitol, adonitol, and salicin The results obtained from l-arabinose, d-xylose d galactose maltose and d glycerol are presented in *Table 1*

Acid formation was seen somewhat slower (2 5 days) from d-galactose, and from l-arabinose The results obtained with maltose differed with the incubation temperature, acid being formed rapidly at 22° C but late (10 30 days) or not at all at 37° C Acid was also formed rapidly from glycerol at 22° C but somewhat later (2–5 days) at 37° C

The old strains of animal origin behaved in the same way as the human strains except concerning d-xylose At 22° C all chinchilla strains formed acid rapidly from this sugar but rather late at 37° C Both recently isolated and old human strains as well as the dog strain showed negative or late and irregular acid production

Slight gas production at 22° C was noted most distinctly from maltose, d mannitol, d glucose, d-trehalose, glycerol, sorbitol and in some cases also from d-xylose Slight gas formation was also noted (though irregularly) from d-glucose at 37° C

TABLE 1

Acid Formation from l Arabinose d Xylose d Galactose Maltose and Glycerol at 22° C and 37° C by 28 strains of Y enterocolitica from Human and Animal Sources

Strain	Origin	l arabinose 22°	l arabinose 37°	d xylose 22°	d xylose 37°	d galactose 22°	d galactose 37°	maltose 22°	maltose 37°	glycerol 22°	glycerol 37°
6498	man	2	2	–	–	3	3	1	10	1	5
4528		2	2		20	3	3	1	20	1	3
5525		2	2			2	2	2		1	5
5674		2	2	–		3	3	1	–	1	5
5253		3	3			3	3	2		1	3
5788	„	2	2			3	3	1		1	2
5944		3	3	–		3	3	1		1	3
6064	„	5	5			5	5	2		1	3
101		5	5		–	3	3	1		1	3
564		2	2	–	–	3	3	1	–	1	3
582		2	2	–	–	3	3	1		1	3
953		3	3		–	3	3	1	10	1	2
1029		3	3		–	5	5	1		1	3
1077		3	3			5	5	1	–	1	2
1168	„	3	3	–		5	3	2	–	1	3
1691		3	3		–	3	3	1		2	3
1759		2	5		–	5	3	1	10	2	3
PY*		2	2		20	3	3	1	10	1	5
336*	„	3	3	–	20	5	5	1	20	1	5
356*	dog	2	2	20	20	3	3	1	10	1	2
334*	chinchilla	1	1	2	20	3	3	1	20	1	5
335*		1	1	1	10	3	3	1	–	1	5
337*		1	1	2	20	3	3	1		1	5
338*		1	1	2	5	3	1	1	10	1	3
339*		3	3	2	20	3	3	1		1	5
347*		1	1	2	20	3	3	1	10	1	5
348*		1	1	1	10	3	3	1	20	1	5
357*		1	1	1	10	3	3	1	10	1	1

The figures indicate the day on which acid formation was registered The tubes were inspected after 1 2 3 5 10 20 and 30 days incubation
– negative reaction after 30 days * old "laboratory" strains

The V P reaction was positive at 22° C but negative at 37° C for all newly isolated human strains

Aesculin splitting was negative in all cases except 2 (strains 357 and 335) where a positive reaction was seen at 37° C after 5 and 7 days respectively

All strains gave a positive urease test most rapidly at 37° C

The ONPG reaction was positive for all strains tested except one (strain 356) Positive reactions were seen earlier at 22° C (after about 3 hours) while at 37° C several strains did not give distinctly positive results before 24 hours in 2 cases (338 5788) not before 48 hours

The catalase reaction was positive in all cases The oxidase reaction was negative All strains reduced nitrate at 22° C and at 37° C Indole and H_2S were not produced

Ornithine decarboxylation was invariably positive strongest at 22° C Arginine and lysine were not decarboxylated

Phenylalanine was not deaminated, and citrate was not utilized at either temperature.

All strains were motile in semisolid agar at 22° C, but not at 37° C.

In tube test O-antigen preparations of all human strains were agglutinated by rabbit immune serum against strain PX at a titre of 1:640–1:1280. O-antigen preparations of the animal strains were agglutinated at approximately the same titres. Only for one strain (357) the O-antigen preparation was agglutinated at a lower titre (1:80–1:160). All strains were distinctly agglutinated on slide agglutination with rabbit antisera, prepared against O-, OH-, and living antigen preparations of strain PX.

TABLE 2

Sensitivity to Antibiotics of 28 Yersinia enterocolitica Strains from Human and Animal Sources Determined by the Paper Disc Method according to Ericsson *1960, 1966*

	I	II	III	IV
Streptomycin	28		–	
Tetracycline	28			
Oxytetracycline	28			
Chloramphenicol	28			
Nitrofurantoin	28			
Colistin	28	–		
Nalidixic acid	28			
Neomycin	25	3		
Sulphonamide	28	–		
Kanamycin	–	28		
Erythromycin	–	–	15	13
Ampicillin	2	1	24	1
Benzylpenicillin		–	14	14
Methicillin	–	–	–	28
Oleandomycin		–	–	28
Novobiocin				28
Fucidin		–	–	28

The figures indicate number of strains within each sensitivity group. I = "sensitive", II = "fairly sensitive", III = "slightly sensitive", IV = "resistant".

Test for Sensitivity to Antibiotics

As shown in Table 2 all strains including the older ones of animal origin showed largely the same pattern of susceptibility to various antibiotics. Thus all strains were sensitive to streptomycin, tetracycline, oxytetracycline, chloramphenicol, nitrofurantoin, colistin, sulphonamide and nalidixic acid. Neomycin gave inhibition zones just below the limit for sensitivity grade I for 3 strains, while the other strains were fully sensitive.

Kanamycin regularly gave inhibition zones corresponding to sensitivity grade II. Sensitivity grade III to erythromycin was seen in 15 cases, 8 of the 13 strains in grade IV showed very small inhibition zones.

All animal strains and 2 recently isolated human strains did not show

any zones for benzyl penicillin while the rest showed small zones corresponding to approximately sensitivity grade III One strain was resistant to ampicillin the values for the others varied from I III All strains were resistant to methicillin fucidin oleandomycin and novobiocin

DISCUSSION

In previous reports the small number of Y e strains of human origin hitherto isolated have not been found to differ substantially from those isolated from various animals (*Hassig et al* 1949 *Carlsson et al* 1964 *Mollaret & Chevalier* 1964) For one strain described by *Wauters & Mollaret* (1965) however aesculin splitting was positive The B enterocoliticum strains reported by *Schleifstein & Coleman* (1939) were atypical in producing indole Two old strains of human origin (33C Hassig PX Winblad) were included in the present study

Differences between strains from different animal species have been reported by *Mollaret & Lucas* (1965) They found strains from hares to differ in certain biochemical properties from strains of other origins

In the present study the only noticeably difference in biochemical behaviour between human strains and old chinchilla strains included in the tests was found in d xylose fermentation Most of the animal strains formed acid rapidly at 22° C while the recently isolated human strains did not (in a few cases very late at 37° C) It must however be borne in mind that the animal strains used in the tests all were laboratory strains in which behavioural changes might have occurred Furthermore other investigators have found strains from various animal sources to differ regarding xylose fermentation (*Becht* 1962 *Mollaret & Chevalier* 1964 *Frederiksen* 1964)

An interesting finding was the variation of biochemical activity of Y e with the incubation temperature Thus diversity in acid formation with greater activity at 22° C than at 37° C was noted from d xylose maltose and glycerol As a rule production of gas from several carbohydrates was noted only at 22° C l ornithine decarboxylation and β galactosidase activity likewise seemed to be more pronounced at the lower incubation temperature Negative V P reactions at 37° C and positive reactions at 22° C were seen in all recently isolated human strains A more detailed study of the V P reaction of Y e will be the subject of a future paper (*Nilehn* to be published) Variation of the activity of Y e with the incubation temperature was also observed in the V P reaction by *Dickinson & Mocquot* (1961 *Frederiksen* (1964) *Mollaret & Chevalier* (1964) and *Wauters & Mollaret* (1965

As far as is known the influence of temperature on growth and various metabolic processes of Y e has not been properly investigated According to *Knapp & Thal* 1963 the optimum temperature for growth is 37° C but they gave no data on the generation time s or growth curves under different conditions It is however well known that se

veral biochemical reactions of bacteria are more intense at temperatures below that for optimal growth

The urease activity was found to be more pronounced at 37° C Preliminary investigations indicate shorter survival times of the bacteria at this temperature This might speak in favour for the existence of a mainly intracellular urease enzyme which is liberated in greater amounts in connection with the disintegration of the cells However, the optimum temperature for the activity of the urease enzyme of this species is not known

Aesculin splitting was seen rather late at 37° C but not at 22° C for 2 animal strains while all human strains were negative at both temperatures.

The above differences found at different incubation temperatures may lead to confusing results in investigations of the biochemical properties of Y e unless the incubation temperature is strictly controlled On the other hand the results of tests carried out at different well controlled temperatures might be valuable in the diagnose of the species

As to the serological testing of the strains by agglutination, the purpose of the present study was not a detailed determination of any of the various antigen components However, at least one O-antigen component was found to be common to all the strains included in the study

The susceptibility pattern of the strains to antibiotics was largely the same as that reported by other investigators (*Frederiksen* 1964 *Smith & Thal* 1965)

SUMMARY

Twenty-eight strains of Yersinia enterocolitica 17 recently isolated from human sources and 11 old strains of animal and human origin were tested biochemically, physiologically and serologically as well as regarding their susceptibility to antibiotics

Differences in the biochemical activity at 37° C and at 22° C were noted in a number of oxidation/fermentation reactions, aesculin hydrolysis, decarboxylase activity β-galactosidase activity, urease activity and in the V P-reaction Recently isolated human strains as well as old human strains differed from old chinchilla strains in respect of d-xylose fermentation

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STUDIES ON TRANSFORMATION IN *MORAXELLA* AND ORGANISMS ASSUMED TO BE RELATED TO *MORAXELLA*

7. *Affinities between Oxidase Positive Rods and Neisseriae as Compared with Group Interactions on Both Sides*

By

KJELL BØVRE

Received 25.xi.66

Relatively efficient genetic transfer has previously been shown to occur between *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying, nonhaemolytic moraxellae (*Bøvre* 1965 a, c). A 19116/51 group of *Moraxella nonliquefaciens*-like organisms has been described (*Bøvre* 1965 d). In quantitative tests the latter group showed no compatibility with the above mentioned moraxellae. However, sensitive tests with continuous DNA exposure revealed low frequent reactions between them.

Low frequent transformation of *Neisseria catarrhalis* with DNAs from *Moraxella nonliquefaciens*, *Moraxella bovis* and *Moraxella liquefaciens* was first reported by *Bøvre* (1963). *Catlin* (1964) has described transformation reactions of similar order between *Neisseria catarrhalis* and some oxidase positive rods probably belonging to the 19116/51 group (*Bøvre* 1965 d).

Observed transformation between *Neisseria catarrhalis*, *Neisseria caviae* and *Neisseria ovis* (*Bøvre* 1965 b) has made also the latter two species natural objects for studies on transformation between oxidase positive rods and neisseriae.

The present report aims at a comparative synthesis of transformation interactions which all turned out as less pronounced than the ones found between *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying, nonhaemolytic moraxellae. Quantitative streptomycin resistance transformation will be supplemented with semiquantitative estimates with the same marker, and a system of transformation with haemolysis as the genetic marker will be introduced.

MATERIAL AND METHODS

All bacterial strains used have been described in previous reports (*Bøvre* 1964 b, 1965 a, b, c, d). Individual strains were chosen in accordance with results of trans-

formation experiments (loc cit) to secure that they were representative of the various groups in question

The methodology of quantitative streptomycin resistance transformation was that initially described (*Bovre* 1964 a) with slight modifications and control measures as presented in the subsequent reports cited above Recipient population densities generally ranged from 10 to 10^9 colonyforming units per ml and 40 µg of DNA was employed per ml of the transformation mixture

Continuous DNA exposure (without termination of DNA action by means of DNase) was used extensively The semiquantitative application of this technique may have different purposes (*Bovre* 1964 b 1965 a b c) Because of its high sensi with a view to those transformation quantified in 15 min DNA exposure was principally as follows In experi ments with highly competent recipients the donor capacities of various heterologous organisms were compared with each other If the activity of one or more of the donors happened to be just quantifiable in 15 min DNA exposure continuous DNA exposure permitted rough translation into quantitative terms of parallel ob servations (see section of results and *Bovre* 1967)

All parallels to be compared in the continuous DNA exposure experiments con sisted of aliquots of the same recipient population simultaneously exposed to the same quantities of the various DNA extracts under identical conditions The DNA preparations had been subjected to the same extraction process (*Bovre* 1964 a) and were measured for total DNA content by means of Dische s diphenylamine method Recipient populations and DNA extracts were mixed in fluid transformation medium (*Bovre* 1964 a) Sometimes mixing was performed in an ice water bath and plating immediately thereafter In other cases the mixtures were placed in the cold for plating after identical periods (15–30 min) at 32–33° C Usually 40 µg of DNA was

at the same temperature and humidity before use

The mutant DNA extracts were all prepared from the same mutant isolates as those used in previous investigations The DNase sensitivity of the interactions to be reported was under control

Transformation with DNA extracts prepared from first round transformants was performed by the usual quantitative streptomycin resistance transformation tech nique Transformants employed as source of DNA had in general been selected at 50 µg of streptomycin per ml but were all able to grow uniformly at 1000 µg of strept mycin per ml and on media without streptomycin as shown by velvet replica plating and by ordinary surface streaking

Transformation with haemolysis as the genetic marker was employed parallel to streptomycin resistance transformation in some instances using the same quantita tive method with the exception that streptomycin addition was omitted in those parallels intended for assay of haemolytic transformants The assay medium was human blood agar (*Bovre* 1964 a) As in the case of streptomycin resistant trans formants the occurrence exclusively in surface cultures made assay and control of the haemolytic colonies or plaques easy This was performed either by direct in spection of the plates study of the blood agar after the colonies had been removed secondary spreading of suspected plaques or by combinations of these methods

RESULTS

Streptomycin Resistance Transformation

In Table 1 are presented results of quantitative and semiquantitative transformation to streptomycin resistance of various ... positive

TABLE 1

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and Neisseriae with Rods as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastrain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure at µg per ml of streptomycin		
				10	50	500
1	5873	N *flavescens* 13120	$<2.4 \cdot 10^{-5}$	0	0, 0	0
		N *catarrhalis* Ne11	$<2.4 \cdot 10^{-5}$	30	32, 37	28
		N *catarrhalis* 4103	$<2.4 \cdot 10^{-5}$	18	50, 66	32
		N *ovis* 199/55	$<2.4 \cdot 10^{-5}$	49	56, 43	47
		M *nonliq* 7784		39	44, 51	45
		M *nonliq* 4663/62		60	72, 95	83
2	5873	M *nonliq* 4663/62			81, 100	
		M *bovis* 10900			89, 121	
		M *liquefaciens* 9833	$4.0 \cdot 10^{-5}$		314, 332	
3	M *nonliq* 7784	N *flavescens* 13120	$<2.5 \cdot 10^{-5}$	0	0	0
		N *catarrhalis* Ne11	$<2.5 \cdot 10^{-5}$	74	21	0
		N *catarrhalis* 4103	$<2.5 \cdot 10^{-5}$	73	68	16
		N *ovis* 199/55	$<2.5 \cdot 10^{-5}$	500*	500	21
		5873		16	15	3
		8134		29	27	9
		8292		16	16	7
		19116/51		18	18	2
4	M *nonliq* 4663/62	N *flavescens* 13120	$<2.9 \cdot 10^{-5}$	0	(4)§	0
		N *catarrhalis* Ne11	$<2.9 \cdot 10^{-5}$	0	(1)§	0
		N *catarrhalis* 4103	$<2.9 \cdot 10^{-5}$	41	55	3
		N *ovis* 199/55	$<2.9 \cdot 10^{-5}$	600*	600	6
		5718		8	8	2
		5833		58	51	15
		9893		27	40	11
		10973		16	30	15
		A608		51	56	10
		A1920		31	30	28
5	(M *lacunata*) A947(1)	N *catarrhalis* Ne11	$<5.9 \cdot 10^{-6}$	0	0	
		N *ovis* 199/55	$2.9 \cdot 10^{-5}$	73	87, 66	
		8292	$<5.9 \cdot 10^{-6}$	10	11, 5	
6	M *bovis* 10900	N *caviae* 14659	$<1.6 \cdot 10^{-4}$	122	112	98
		N *ovis* 199/55	$<1.6 \cdot 10^{-4}$	24	20	53
7	M *bovis* 10900	N *catarrhalis* Ne11		3	3	0
		N *ovis* 199/55			225	234
		8292		16	18	37

N = Neisseria M = Moraxella nonliq = *nonliquefaciens* Strains with no species designation belong to the 19116/51 group (*Bøvre* 1965 d) (M *lacunata*) See *Bøvre* (1965 c)
Experiment no refers to the semiquantitative part of the table (continuous DNA exposure) The latter results can be directly compared only within each experiment All single plate counts given The results of 15 min DNA exposure were obtained simultaneously with the semiquantitative results or in separate experiments See preceding communications for intrastrain transformability and calculation of ratios $<$ = less than 1 interstrain transformant per 0.1 ml at uncritical streptomycin concentration * Approximate figures § Probably mutants

rods with DNAs of streptomycin resistant mutants of neisseriae and oxidase positive rods The results of quantitative experiments may be supplemented with previously performed transformation between the 19116.51 group and other oxidase positive rods (*Bovre* 1964 b, 1965 a c d) In the semiquantitative tests transformants are noted in most of the combinations contrary to the results with 15 min DNA exposure That some reactivity is observed between representatives of groups which previously have shown less striking or no interaction is not surprising in view of the following facts (1) Slight differences in donor efficiency towards a given heterologous recipient may be seen within a group of closely interrelated donors (2) Competence of a recipient strain may vary from time to time (3) Recipients of the same group are not equally potent (4) The sensitivity of the semiquantitative methodology has been improved

Table 1 shows that with some recipients the yield of transformants is critically influenced by the highest selection concentration of streptomycin (500 μg per ml) After replication of transformant assay plates by the velvet method to plates with 500 and 1000 μg of streptomycin per ml practically all colonies assayed at low concentrations also grew secondarily at the high concentrations Exceptional in this respect were approximately half of the transformants elicited at 10 μg by the *Neisseria catarrhalis* Ne 11 donor in experiment no 3 Low yield at the 500 μg concentration may well reflect mechanisms shared by *Haemophilus* (*Alexander & Leidy* 1953) In the latter study low transformant counts at high selection concentration reached the higher level obtained at low concentration when the phenotypic expression period was prolonged *Fox* (1959) concluded on the basis of studies in pneumococci that such findings almost entirely can be accounted for by a delay in action of streptomycin at low concentrations

Indirect expression in quantitative terms of results in continuous DNA exposure may be illustrated by means of experiments no 1 and 2 of Table 1 The average 323 transformants elicited by *Moraxella liquefaciens* in the "19116.51 group recipient of experiment no 2 are equivalent to the ratio 4 0 10^{-5} of inter to intrastrain transformation Then the 105 and 91 transformants elicited by *Moraxella bovis* and *Moraxella nonliquefaciens* in the same experiment are considered equivalent to a transformation compatibility of the order 1 10^{-5} By extrapolating this value into experiment no 1 the donor *Moraxella nonliquefaciens* 7784 reveals the approximate ratio 6 10^{-6} with the "19116.51 group recipient

Generally *Neisseria* [illegible] and *Neisseria catarrhalis* 4103 reveal at least as high reactivity with the rods as the 19116.51 group shows with other oxidase positive rods (Table 1) This activity between rods and cocci seems particularly pronounced between the *Neisseria* [illegible] as donor and the recipients recognized as unequivocal moraxellae It is important to note here that *Neisseria catarrhalis* has an even greater reactivity to

TABLE 2

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and Neisseriae with Neisseriae as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastrain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure
1	*N catarrhalis* Ne11	5873	< 9.9 10 r	64
		A1920		186
		8292	4.0 10⁻⁵	410
		M nonliq 5030/62	3.8 10⁻⁵	
		M nonliq 5058/62	3.8 10'	
		M nonliq 13536/62	3.5 10⁻⁶	
		M nonliq 7784	5.0 10⁻⁶	
			1.8 10 ∘	
		M nonliq 4663/62	5.9 10⁻⁵	825
			7.4 10⁻⁶	
		M bovis 10900	5.0 10⁻⁵	862
			2.3 10⁻⁵	
		M liquefaciens 9833	5.0 10 ∘	892
		N ovis 199/55	3.4 10 ∘	
2	*N catarrhalis* 4103	5873	< 1.5 10⁻⁵	406
		A1920		157
		8292		408
		M nonliq 7784	< 1.5 10⁻⁶	
		M nonliq 4663/62	< 1.5 10⁻⁵	194
		M bovis 10900	< 1.5 10⁻⁵	530
		M liquefaciens 9833	< 1.5 10⁻⁵	590
3	*N ovis* 199/55	5873	2.2 10⁻⁶	22
			3.1 10⁻⁵	
		A1920		233
		8292	4.4 10⁻⁵	613
		M nonliq 7784	7.5 10⁻⁵	
			8.6 10⁻⁶	
		M nonliq 4663/62	1.0 10⁻⁴	2200
			9.0 10⁻⁵	
		M bovis 10900	6.6 10⁻⁴	10000
			6.2 10⁻⁴	
			8.4 10⁻⁴	
			1.1 10⁻³	
		M liquefaciens 9833	2.4 10⁻⁴	5000
		N catarrhalis Ne11	4.6 10⁻⁵	

Strain designations As in legend of *Table 1*
Experiment no refers to the semiquantitative part of the table (continuous DNA exposure All figures based on at least two plate counts at 50 µg of streptomycin per *ml* (uncritical) The results of 15 *min* DNA exposure were obtained in separate experiments See preceding communications for intrastrain transformability and calculation of ratios < = less than 1 transformant per 0 1 ml * Approximate figures

wards the *Moraxella bovis* recipient than the *Neisseria ovis* donor *Neisseria catarrhalis* Ne 11 shows irregular activity which may be a reflection of slightly lower resistance of transformants than usual *Neisseria flavescens* is inactive in transformation of oxidase positive

rods, as it was towards *Neisseria catarrhalis* and *Neisseria ovis* recipients (*Bovre* 1965 b).

In Table 2 are presented similar experiments with *Neisseria* recipients and oxidase positive rods as donors, in part compared with heterologous *Neisseria* donor activities (newly obtained values, for comparison see *Bovre* 1965 b). The efficiency of transformation in this direction between rods and cocci appears greater than in the reverse reactions of Table 1. The reactions have been quantifiable in 15 min DNA exposure in most cases and the counts in continuous exposure are high. In fact, the reactions of experiments similar to experiment no. 1 of Table 2 were so high frequent that the sensitivity of continuous DNA exposure had to be geared down by using a dilute recipient population. Thousands of colonies per plate were obtained in experiments not tabulated.

Generally, the compatibilities of the *Neisseria catarrhalis* Ne 11 recipient with the oxidase positive rods are of the same order as between the same recipient and *Neisseria ovis* (Table 2). Between the *Neisseria ovis* recipient and the oxidase positive rods the affinities in these terms are partly much greater than between *Neisseria ovis* and *Neisseria catarrhalis*. Particularly the reactions of *Neisseria ovis* with *Moraxella bovis* are of a relatively high order, with ratios of inter- to intrastrain transformation ranging from $6.2 \cdot 10^{-4}$ to $1.1 \cdot 10^{-3}$. Donors of the "19116/51" group of oxidase positive rods appear least effective towards *Neisseria catarrhalis* Ne 11 (with indirectly calculated ratios down to $6 \cdot 10^{-6}$) and towards the *Neisseria ovis* recipient (with ratios down to $2.2 \cdot 10^{-5}$). The *Neisseria catarrhalis* 4103 recipient does not distinguish between the donors in the same manner and seems less receptive (for the deviation of strain 4103 from typical *Neisseria catarrhalis* strains, see *Bovre* 1965 b). The relatively low donor efficiency of members of the '19116/51' group on typical *Neisseria catarrhalis* organisms was also shown in a not tabulated experiment where *Neisseria catarrhalis* 8176 was transformed more efficiently by *Moraxella nonliquefaciens*, *Moraxella bovis* and *Moraxella liquefaciens* than by the "19116/51" group donor 5893.

Although irregularities are observed, the results of Table 2 generally point to consistency of relative affinities as determined in 15 min DNA exposure and continuous exposure. This tendency, particularly seen in experiment no. 3, is considered of great importance for deductions from continuous exposure experiments.

The interactions found between the *Neisseria ovis* recipient and *Moraxella bovis* are more frequent than those previously described between *Neisseria ovis* and *Neisseria caviae* (*Bovre* 1965 b). The results presented in Table 3, where the effects on *Neisseria ovis* of three *Moraxella bovis* donors and two *Neisseria caviae* donors are compared in the same experimental setup, are corroborative on this point.

From representative heterologous combinations listed in Tables 1

TABLE 2

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and Neisseriae with Neisseriae as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter- to intrastrain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure
1	*N catarrhalis* Ne11	5873	$<9.9 \times 10^{-6}$	64
		41920		136
		8292	4.0×10^{-5}	410
		M nonliq 5050 62	3.8×10^{-5}	
		M nonliq 5058 62	3.8×10^{-5}	
		M nonliq 13536 62	3.5×10^{-5}	
		M nonliq 7784	5.0×10^{-5}	
			1.8×10^{-5}	
		M nonliq 4663 62	5.9×10^{-5}	525
			7.4×10^{-5}	
		M bovis 10900	5.0×10^{-5}	862
			2.3×10^{-5}	
		M liquefaciens 9833	5.0×10^{-5}	[illegible]
		N ovis 19955	3.4×10^{-5}	
2	*N catarrhalis* 4103	5873	$<1.5 \times 10^{-5}$	406
		41920		15
		8292		408
		M nonliq 7784	$<1.5 \times 10^{-5}$	
		M nonliq 4663 62	$<1.5 \times 10^{-5}$	194
		M bovis 10900	$<1.5 \times 10^{-5}$	530
		M liquefaciens 9833	$<1.5 \times 10^{-5}$	520
3	*N ovis* 19955	5873	2.2×10^{-5}	22
			3.1×10^{-5}	
		41920		23
		8292	4.4×10^{-5}	61
		M nonliq 7784	7.5×10^{-5}	
			8.6×10^{-5}	
		M nonliq 4663 62	1.0×10^{-4}	2200
			9.0×10^{-5}	
		M bovis 10900	6.6×10^{-4}	10000
			6.2×10^{-4}	
			8.4×10^{-4}	
			1.1×10^{-5}	
		M liquefaciens 9833	2.4×10^{-4}	500
		N catarrhalis Ne11	4.6×10^{-5}	

Strain designations As in legend of Table 1
Experiment no refers to the semiquantitative part of the table (continuous DNA exposure). All figures based on at least two plate counts at 50 µg of streptomycin per ml (uncritical). The results of 15 min DNA exposure were obtained in separate experiments. See preceding communications for intrastrain transformability and calculation of ratios < = less than 1 transformant per 0.1 ml * Approximate figures

wards the *Moraxella bovis* recipient than the *Neisseria ovis* donor *Neisseria catarrhalis* Ne 11 shows irregular activity which may be a reflection of slightly lower resistance of transformants than usual *Neisseria flavescens* is inactive in transformation of oxidase positive

rods as it was towards *Neisseria catarrhalis* and *Neisseria ovis* recipients (*Bovre* 1965 b).

In Table 2 are presented similar experiments with *Neisseria* recipients and oxidase positive rods as donors in part compared with heterologous *Neisseria* donor activities (newly obtained values for comparison see *Bovre* 1965 b). The efficiency of transformation in this direction between rods and cocci appears greater than in the reverse reactions of Table 1. The reactions have been quantifiable in 15 min DNA exposure in most cases and the counts in continuous exposure are high. In fact the reactions of experiments similar to experiment no. 1 of Table 2 were so high frequent that the sensitivity of continuous DNA exposure had to be geared down by using a dilute recipient population. Thousands of colonies per plate were obtained in experiments not tabulated.

Generally the compatibilities of the *Neisseria catarrhalis* Ne 11 recipient *with the oxidase positive rods are of the same order as between* the same recipient and *Neisseria ovis* (Table 2). Between the *Neisseria ovis* recipient and the oxidase positive rods the affinities in these terms are partly much greater than between *Neisseria ovis* and *Neisseria catarrhalis*. Particularly the reactions of *Neisseria ovis* with *Moraxella bovis* are of a relatively high order with ratios of inter- to intrastrain transformation ranging from $6.2 \cdot 10^{-4}$ to $1.1 \cdot 10^{-3}$. Donors of the 19116/51 group of oxidase positive rods appear least effective towards *Neisseria catarrhalis* Ne 11 (with indirectly calculated ratios down to $6 \cdot 10^{-6}$) and towards the *Neisseria ovis* recipient (with ratios down to $2.2 \cdot 10^{-5}$). The *Neisseria catarrhalis* 4103 recipient does not distinguish between the donors in the same manner and seems less receptive (for the deviation of strain 4103 from typical *Neisseria catarrhalis* strains see *Bovre* 1965 b). The relatively low donor efficiency of members of the "19116/51" group on typical *Neisseria catarrhalis* organisms was also shown in a not tabulated experiment where *Neisseria catarrhalis* 8176 was transformed more efficiently by *Moraxella nonliquefaciens*, *Moraxella bovis* and *Moraxella liquefaciens* than by the 19116/51 group donor 5893.

Although irregularities are observed the results of Table 2 generally point to consistency of relative affinities as determined in 15 min DNA exposure and continuous exposure. This tendency particularly seen in experiment no. 3 is considered of great importance for deductions from continuous exposure experiments.

The interactions found between the *Neisseria ovis* recipient and *Moraxella bovis* are [illegible] frequent than those previously described between *Neisseria* [illegible] and *Neisseria ca*[illegible] (*Bovre* 196[illegible]). The

TABLE 2

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and Neisseriae with Neisseriae as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastrain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure
1	*N catarrhalis* Ne11	5873	$<9\ 9\ 10^{6}$	64
		A1920		186
		8292	$4\ 0\ 10^{5}$	410
		M nonliq 5050/62	$3\ 8\ 10^{5}$	
		M nonliq 5058 62	$3\ 8\ 10^{5}$	
		M nonliq 13536/62	$3\ 5\ 10^{5}$	
		M nonliq 7784	$5\ 0\ 10^{5}$	
			$1\ 8\ 10^{5}$	
		M nonliq 4663/62	$5\ 9\ 10^{5}$	8?5
			$7\ 4\ 10^{5}$	
		M bovis 10900	$5\ 0\ 10^{5}$	86?
			$2\ 3\ 10^{5}$	
		M liquefaciens 9833	$5\ 0\ 10^{5}$	832
		N ovis 199/55	$3\ 4\ 10^{5}$	
2	*N catarrhalis* 4103	5873	$<1\ 5\ 10^{5}$	406
		A1920		157
		8?92		408
		M nonliq 7784	$<1\ 5\ 10^{5}$	
		M nonliq 4663 62	$<1\ 5\ 10^{5}$	194
		M bovis 10900	$<1\ 5\ 10^{5}$	530
		M liquefaciens 9833	$<1\ 5\ 10^{-5}$	520
3	*N ovis* 199 55	5873	$2\ ?\ 10^{5}$	2?7
			$3\ 1\ 10^{5}$	
		A1920		233
		8292	$4\ 4\ 10^{-5}$	613
		M nonliq 7784	$7\ 5\ 10^{5}$	
			$8\ 6\ 10^{5}$	
		M nonliq 4663/62	$1\ 0\ 10^{4}$	~200
			$9\ 0\ 10^{5}$	
		M bovis 10900	$6\ 6\ 10^{4}$	10000*
			$6\ ?\ 10^{4}$	
			$8\ 4\ 10^{4}$	
			$1\ 1\ 10^{3}$	
		M liquefaciens 9833	$2\ 4\ 10^{4}$	5000
		N catarrhalis Ne11	$4\ 6\ 10^{5}$	

Strain designations As in legend of Table 1
Experiment no refers to the semiquantitative part of the table (continuous DNA exposure) All figures based on at least two plate counts at 50 µg of streptomycin per ml (uncritical) The results of 15 min DNA exposure were obtained in separate experiments See preceding communications for intrastrain transformability and calculation of ratios < = less than 1 transformant per 0 1 ml * Approximate figures

wards the *Moraxella bovis* recipient than the *Neisseria ovis* donor *Neisseria catarrhalis* Ne 11 shows irregular activity which may be a reflection of slightly lower resistance of transformants than usual *Neisseria flavescens* is inactive in transformation of oxidase positive

Streptomycin Resistance Transformation with DNAs Extracted from Transformants

Recipient strain	Donor strain	Origin of marker in donor strain*	DNA extract		Relative transformation frequency
5873	*M nonliq* 7784	5873	(7784Smr5873)	no 1	2 0 10^{4}
				no 2	3 0 10^{3}
				no 3	3 4 10^{4}
	N catarrhalis Ne11		(Ne11Smr5873)	no 1	< 4 8 10^{6}
				no 2	< 2 4 10^{5}
				no 3	< 4 8 10^{6}
	N catarrhalis 4103		(4103Smr5873)	no 1	3 6 10^{4}
				no 2	1 4 10^{3}
				no 3	1 0 10^{3}
	N ovis 199/55		(199/55Smr5873)	no 1	1 7 10^{2}
				no 2	2 3 10^{2}
				no 3	4 1 10^{4}
	5873		(5873Smr5873)		1 (0 4%)§
M nonliq 7784	5873	*M nonliq* 7784	(5873Smr7784)	no 1	1 3 10^{2}
				no 2	1 2 10^{2}
				no 3	1 1 10^{2}
	N catarrhalis Ne11		(Ne11Smr7784)	no 1	1 9 10^{5}
				no 2	1 9 10^{5}
				no 3	1 1 10^{4}
	N catarrhalis 4103		(4103Smr7784)	no 1	3 1 10^{4}
				no 2	3 6 10^{4}
				no 3	3 2 10^{3}
	N ovis 199/55		(199/55Smr7784)	no 1	1 5 10^{2}
				no 2	3 6 10^{3}
				no 3	3 1 10^{3}
	M nonliq 7784		(7784Smr7784)		1 (0 5 0 9%)§
M bovis 10900	*N ovis* 199/55	*M bovis* 10900	(199/55Smr10900)		4 3 · 10^{2}
	M bovis 10900		(10900Smr10900)		1 (0 03%)§

M — *Moraxella*, *nonliq* — *nonliquefaciens*, *N* — *Neisseria*. Strain 5873 belongs to the '19116/51' group (*Bøvre* 1965 d).
15 min DNA exposure. Selection of transformants at 50 µg and sometimes also at 10 µg of streptomycin per ml. Figures based on means of two plate counts.
* Marker introduced in donor strain in a previous transformation.
§ Unit for expression of relative transformation frequency. In brackets: Percentage of recipient population transformed. The effect of intrastrain transformant DNA did not differ significantly from that of mutant DNA.

TABLE 5

Streptomycin Resistance Transformation with DNAs Extracted from Transformants

Recipient strain	Donor strain	Origin of marker in donor strain*	DNA extract		Relative transformation frequency
N catarrhalis Ne11	5873	*N catarrhalis* Ne11	($5873Sm^rNe11$)	no 1	5 9 10^{-2}
				no 2	8 9 10^{-2}
				no 3	1 2 10^{-1}
	M nonliq 7784		($7784Sm^rNe11$)	no 1	1 5 10^{-2}
				no 2	<2 9 10^{-5}
				no 3	6 7 $10^{-?}$
	N catarrhalis Ne11		($Ne11Sm^rNe11$)		1 (0 9 3 9%)§
N catarrhalis 4103	5873	*N catarrhalis* 4103	($5873Sm^r4103$)	no 1	1 1 10^{-1}
				no 2	1 5 10^{-1}
	M nonliq 7784		($7784Sm^r4103$)	no 1	1 1 10^{-3}
				no 2	8 7 10^{-4}
				no 3	1 3 10^{-3}
	N catarrhalis 4103		($4103Sm^r4103$)		1 (0 5%)§
N ovis 199/55	5873	*N ovis* 199/55	($5873Sm^r199/55$)	no 1	2 6 10^{-2}
				no 2	2 4 10^{-2}
	M nonliq 7784		($7784Sm^r199/55$)	no 1	1 4 $10^{-[illegible]}$
				no 2	7 4 10^{-3}
				no 3	2 3 10^{-2}
	M bovis 10900		($10900Sm^r199/55$)	no 1	2 3 10^{-2}
				no 2	1 3 10^{-2}
				no 3	1 2 10^{-3}
	N ovis 199/55		($199/55Sm^r199/55$)		1 (0 5%)§

Legend identical with that of Table 4

TABLF 6

Streptomycin Resistance Transformation with DNAs Extracte l from Transformar ts

Recipient *strain*	D nr r strain	Origin of marker in *donor strain**	DNA extract		Relative transformation frequency
5873	5873	*M nonliq* 7784	(5873Smr7784)	no 3	1 1 10^{0}
		N catarrhalis Ne11	(5873SmrNe11)	no 3	7 5 10^{1}
		N catarrhalis 4103	(5873Smr4103)	no 2	1 2 10^{0}
		N ov is 199/55	(5873Smr199/55)	no 1	7 9 10^{-1}
		5873	(5873Smr5873)		1 (0 4 0 5%)§
M nonliq 7784	*M nonliq* 7784	5873	(7784Smr5873)	no 2	9 0 10^{1}
		M nonliq 7784	(7784SmrNe11)	no 3	6 5 10^{1}
		N catarrhalis 4103	(7784Smr4103)	no 3	1 0 10^{0}
		N ov is 199/55	(7784Smr199/55)	no 3	8 9 10^{1}
		M nonliq 7784	(7784Smr7784)		1 (0 8%)§
N catarrhalis Ne11	*N catarrhalis* Ne11	5873	(Ne11Smr5873)	no 3	9 6 10^{1}
		M nonliq 7784	(Ne11Smr7784)	no 1	8 6 10^{1}
				no 3	9 7 10^{1}
		N catarrhalis Ne11	(Ne11SmrNe11)		1 (1 2%)§
N catarrhalis 4103	*N catarrhalis* 4103	5873	(4103Smr5873)	no 2	7 4 10^{1}
		M nonliq 7784	(4103Smr7784)	no 3	1 1 10^{0}
		N catarrhalis 4103	(4103Smr4103)		1 (1 0%)§
N ovis 199/55	*N ovis* 199/55	5873	(199/55Smr5873)	no 2	7 6 10^{1}
		M nonliq 7784	(199/55Smr7784)	no 1	9 2 10^{1}
		M bovis 10900	(199/55Smr10900)		9 6 10^{1}
		N ovis 199/55	(199/55Smr199/55)		1 (1 4%)§

Legend identical with that of Table 4

rologous factor) The results are presented in Table 6 The reactions are all of a very high efficiency (lowest value obtained 6 5 10 ¹ of strictly homologous transformation frequency) Consequently, heterologies of the marker sites and adjacent parts of the genomes do not appear to have been of importance for reduction below a first degree compatibility (*Bovre* 1964 b) of the primary transformation reactions between the actual microbes listed in Tables 1 and 2 Similar conclusions have been made on the basis of transformation within *Moraxella (Bovre* 1965 a, c), within *Neisseria* (*Bovre* 1965 b), and also in *Bacillus (Marmur, Seaman & Levine* 1963)

Plates from almost all positive experiments noted in Tables 2 3, 4, 5 and 6 were replicated to plates with 1000 μg of streptomycin per ml With very few exceptions the transformant colonies grew at this concentration Exceptional were transformants with streptomycin resistance marker from *Neisseria catarrhalis* Ne 11, which in some instances tolerated only the next lower concentration tested (500 μg per ml)

First round transformants of all combinations used for DNA extraction were examined for eventual spontaneous loss of the transformed character after lyophilization and several subcultures in media devoid of streptomycin Approximately 50 000 single colonies were studied in simultaneous replica plating to media without streptomycin and supplied with 50 μg of streptomycin per ml No sign of instability was observed

Transformation of Haemolysis

In Figs 1 and 2 is presented the morphology of haemolytic zones on human blood agar produced by *Neisseria ovis* and *Moraxella bovis* colonies The figures are directly comparable

The first indication of genetic transfer of haemolysis from *Moraxella bovis* to *Neisseria ovis* was due to a chance observation on a plate with 10 000 heterologous streptomycin resistant transformants Several plaques of clear haemolysis could be seen on the background of diffuse but less penetrating *Neisseria ovis* haemolysis Secondary culture from the center of some of these plaques revealed *Neisseria ovis* colonies with accentuated haemolysis as shown in Fig 3 Subsequent attempts at exact determination of the transformation frequency were unsuccessful, because of the background haemolysis of the recipient

Fortunately, a completely nonhaemolytic mutant of *Neisseria ovis* 199 55 was available again due to a chance observation at an earlier occasion This mutant was employed as recipient in the quantitative transformation experiments presented in Table 7 Donors were ordinary streptomycin resistant mutants of *Neisseria ovis* and *Moraxella bovis* The haemolysis of *Moraxella bovis* 9425 was as shown in Fig 2 Haemolytic transformants elicited by the *Neisseria ovis* donor were exclusively of the type shown in Fig 1, and those elicited by *Moraxella*

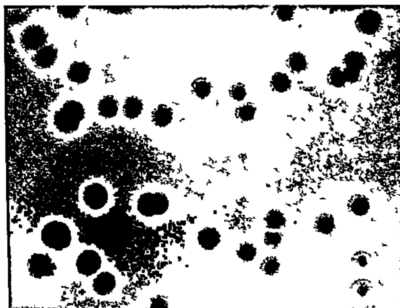


Fig 1

Neisseria ovis 199/55 haemolytic donor



Fig 2

Moraxell b s 10900 haem l   1 n

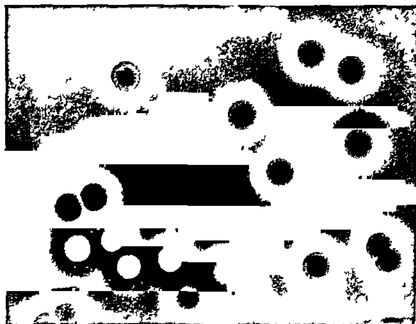


Fig 3

Haemolysis in *Neisseria ovis* 199/55 after transformation of its nonhaemolytic mutant with *Moraxella bovis* 10900 DNA

bovis donors were mainly of the type shown in Fig 3 However approximately 10 per cent of the haemolytic transformants elicited by strain 10900 were of the Fig 1 type

Streptomycin resistant and haemolytic transformants were assayed separately but in parallel under exactly comparable conditions Double transformants were assayed on the plates with streptomycin addition

It can be seen from Table 7 that the frequencies of streptomycin resistance and haemolysis transformation are of approximately the same order in homologous reactions In heterologous reactions however the transformation with haemolysis as the genetic marker is much more efficient than with the streptomycin resistance marker

The experiments of Table 7 show that 40 μg of DNA per ml of the transformation mixture which is now the routine concentration is saturating with respect to both markers even with a very dense population of highly competent recipient bacteria The relative number of double transformants is small and the decreasing proportion of double transformants with decreasing concentration of DNA below saturation further indicates that eventual linkage between the two markers in *Neisseria ovis* must be of low degree (see *Goodgal* 1961 *Goodgal & Herriott* 1961 *Hayes* 1964)

Homologous or heterologous DNA which had been treated with DNase beforehand never gave rise to haemolytic colonies Occurrence of spontaneous haemolytic back mutants of the nonhaemolytic recipi

ent was never observed Some 11 000 widely separated transformant colonies did not reveal signs of spontaneous loss or other instability of the transformed haemolytic character

TABLE 7

Transformation of a Nonhaemolytic Mutant of Streptomycin Sensitive Neisseria ovis 199 55 to Haemolysis and Streptomycin Resistance with Doubly Marked Neisseria ovis and Moraxella bovis DNAs

Donor	Experiment no	Recipient count/ml	μg of DNA/ml	Streptomycin resistant transformants Total count/ml	Per cent haemolytic clones	Haemolytic transformants/ml
N ovis 199/55	1	9.8×10^{8}	40	3.8×10^{6}	0.53	2.9×10^{6}
	2	4.8×10^{8}	40	1.9×10^{6}		1.6×10^{6}
	3	1.7×10^{9}	40	5.0×10^{6}		6.0×10^{6}
	4	2.3×10^{9}	80	5.4×10^{6}		8.8×10^{6}
			40	6.4×10		8.7×10^{6}
			20	5.9×10^{6}		8.2×10^{6}
			10	3.1×10^{6}		4.5×10^{6}
	5	2.3×10^{9}	10	9.6×10^{5}	0.16	
			1	5.3×10	0.13	
			0.02	2.0×10^{5}	0.04	
M bovis 10900	6	5.0×10^{8}	40	5.2×10^{3}		1.5×10^{5}
	3	1.7×10^{9}	40	5.4×10^{3}		2.8×10^{5}
	4	2.3×10^{9}	80	4.7×10^{3}		3.3×10^{5}
			20	4.8×10^{3}		3.2×10
			10	5.0×10^{3}		3.1×10^{5}
			2	3.1×10^{3}		1.7×10^{5}
M bovis 9425	7	8.7×10^{8}	40	1.1×10^{3}		1.0×10^{5}

15 min DNA exposure Selection of streptomycin resistant transformants at 50 μg of streptomycin per ml Figures based on means of two plate counts All observations within one experiment are simultaneous and directly comparable Experiments no 3 and 4 include both homologous and heterologous donors

Attempts at transformation to haemolysis of two strains of *Moraxella nonliquefaciens* and one strain of *Moraxella liquefaciens* with *Moraxella bovis* DNA were unsuccessful in experiments where simultaneous streptomycin resistance transformation showed high activity Transformation of *Neisseria catarrhalis* to haemolysis with *Neisseria ovis* DNA was also unsuccessful On the other hand the weak haemolysis of *Neisseria caviae* seemed to be transferred at high frequency to the nonhaemolytic *Neisseria ovis* recipient Assays of these transformants was very difficult however and exact values cannot be given

DISCUSSION AND CONCLUSION

The relations in terms of streptomycin resistance transformation between *Neisseria catarrhalis* and oxidase positive rods previously re

ported (*Bovre* 1963 *Catlin* 1964) are verified The 19116/51 group seems to be situated at the lower part of the range of compatibility between oxidase positive rods and *Neisseria catarrhalis* It must be noted however that assumed members of the 19116/51 group in the hands of *Catlin* (1964) were more active towards other *Neisseria catarrhalis* recipients than the strain Nc 11 used here It is most valuable that Catlin in the same studies found time and DNA concentration response curves which are consistent with the transformation nature of the change to streptomycin resistance in these interactions

There are some very low frequent interactions between the 19116/51 group and the other oxidase positive rods These interactions are generally no more frequent than those observed when some neisseriae act as donors on rod recipients and are exceeded by the action of *Neisseria ovis* (and *Neisseria caviae*) on unequivocal *Moraxella* recipients

The main outcome of this part of the studies is the relatively pronounced compatibility in streptomycin resistance transformation observed between *Neisseria ovis* and *Moraxella bovis* These interactions are even more frequent than those found between *Neisseria ovis* and *Neisseria caviae* in one way experiments The transformation of haemolysis at high frequency between *Moraxella bovis* and *Neisseria ovis* also indicates that there are particular relations between these organisms Taxonomically important affinities thus seem to exist between *Moraxella* and *Neisseria* as suggested by *Henriksen* (1952) *Moraxella liquefaciens* and *Moraxella nonliquefaciens* are not very far removed from *Moraxella bovis* as regards transformation compatibility with *Neisseria ovis* The 19116/51 group is apparently more distantly related to *Neisseria ovis* than the oxidase positive rods mentioned

It is generally felt that the transformation reactions of the 19116/51 group with other oxidase positive organisms need further evaluation for eventual positive deductions on relationship The same holds for the interactions between *Neisseria catarrhalis* and the oxidase positive rods in general although most of these compatibilities are comparable to that of *Neisseria catarrhalis* with *Neisseria ovis*

As expected from its incompatibility with *Neisseria catarrhalis* *Neisseria flavescens* is incompatible in transformation also with the oxidase positive rods tested (see *Bovre* 1965 b)

SUMMARY

On the basis of quantitative and semiquantitative streptomycin resistance transformation further evidence has been presented of genetic transfer between oxidase positive rods and asaccharolytic not yellow pigmented neisseriae

No interaction exceeded those previously observed between *Morax*

ella nonliquefaciens, *Moraxella bovis* and the serum liquefying, nonhaemolytic moraxellae

A typical *Neisseria catarrhalis* recipient was transformed with ratios of inter- to intrastrain frequencies ranging fom 1 8 10^{-5} to 7 4 10^{-4} by the moraxellae mentioned and with ratios ranging from 6 10^{-6} to 4 0 10^{-5} by members of the "19116 51" group of oxidase positive rods

Neisseria ovis was transformed to streptomycin resistance by *Moraxella bovis* with the ratios 6 2 10^{-4} to 1 1 10^{-3} of inter to intrastrain transformation Even higher compatibility was observed between the two in transformation with haemolysis as the genetic marker The corresponding ratios ranged from 7 5 10^{-3} to 2 4 10^{-4} in reactions between the same recipient and donors of *Moraxella nonliquefaciens* and *Moraxella liquefaciens* The "19116.51" group was less reactive with *Neisseria ovis*

Under the prevailing conditions, genetic transfer seemed less effective from cocci to rods than *vice versa* Very low frequent interactions were observed between the "19116 51" group and other oxidase positive rods More frequent transformation was observed, however, when *Neisseria ovis* and *Neisseria caviae* acted as donors on unequivocal *Moraxella* recipients *Neisseria catarrhalis* DNAs had some irregular activities on rod recipients whereas *Neisseria flavescens* was consistently negative

The interaction between *Neisseria ovis* and *Moraxella bovis* are considered indicative of taxonomically important relations between *Moraxella* and *Neisseria*

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STUDIES ON TRANSFORMATION IN *MORAXELLA* AND ORGANISMS ASSUMED TO BE RELATED TO *MORAXELLA*

8 *The Relative Position of some Oxidase Negative, Immotile Diplobacilli* (Achromobacter) *in the Transformation System*

By

KJELL BØVRE

Received 25 vi 66

Previous transformation experiments involving oxidase negative rods (*Catlin & Cunningham* 1964) have not clearly indicated reactivity with oxidase positive organisms However, development of an extremely sensitive transformation method (*Bøvre* 1967) has permitted supplementary investigations

The affinities of oxidase negative, strictly aerobic and immotile diplobacilli are considered particularly important for a comparative evaluation of the low compatibilities found between the "19116.51" group and other oxidase positive organisms, and between *Neisseria catarrhalis* and oxidase positive rods (*Bøvre* 1967)

MATERIAL AND METHODS

The oxidase negative immotile diplobacilli were represented by 10 strains of rather wide phenotypic diversity In agreement with Dr S D Henriksen all strains were provisionally called *Achromobacter* The strains are listed in Table 1 The strains 8858 62 742/56 and 2181/60 had been isolated from sputum and strain 17938 from urine (probably contaminant) The source of the remaining strains is unknown

The *Achromobacter* strains were examined by conventional means as described (*Bøvre* 1964 b), with some modifications Tests for growth in citrate media were extended to the use of Simmons Citrate Agar (Difco) in addition to the fluid and solid Koser s medium previously used (*Bøvre* 1965 c) Gelatin liquefaction was substituted for serum liquefaction The medium consisted of 15 per cent gelatin in nutrient broth Acid production fr m glucose was tested for in Hugh & Leifs n s medium (slab culture) The glucose oxi lizing strains were also tested in 1 per cent peptone water with 1 per cent glu ose lactose galacto e xylose arabinose an l 10 per cent lactose respectively Br mothymol blue was the indicator of aci l pr duction The incubation peri ds f r reading of gelatin liquefaction and aci l pr du ti n from carbohydrates are giv n in the legend of Table [illegible]

Various strains [illegible]
present stu ly have [illegible]
of the "19116 51 [illegible]
bacter strains for [illegible]
The following st [illegible]

TABLF 1

Designations and Origin of the Achromobacter Strains

Designation in this report	Other designations	Received from
Achromobacter lwoffi 17985	*Moraxella lwoffi* var *bacteroides* (Institut Pasteur) *Moraxella lwoffi* var *bacteroides* ATCC 17985	Institut Pasteur Paris
Achromobacter lwoffi 17987	*Moraxella lwoffi* var *brevis* NCTC 5867 *Moraxella lwoffi* var *brevis* ATCC 17987	NCTC
Achromobacter lwoffi 881/57		§
Achromobacter lwoffi 8858 62		§
Achromobacter anitratus 8	*Bacterium anitratum* 8 (SSI)	Dr H Lautrop, Copenhagen
Achromobacter anitratus 9	*Bacterium anitratum* 9 (SSI)	Dr H Lautrop, Copenhagen
Achromobacter haemolysans 742/56		§
Achromobacter haemolysans 2408/57		§
Achromobacter haemolysans 2181/60	*Achromobacter haemolyticus* var *alcaligenes* 2181/60 (HIUH)	Dr W Mannheim Heidelberg
Achromobacter haemolysans 17988	*Alcaligenes haemolysans* ATCC 17988	
	Alcaligenes haemolysans 4233 62	§

ATCC = The American Type Culture Collection NCTC = The National Collection of Ty Cultures London (SSI) = State Serum Institute Copenhagen (HIUH) = Hygiene-Institut d Universitat Heidelberg § Isolated in Kaptein W Wilhelmsen og Frues Bakteriologiske Inst Osl

ments *Neisseria meningitidis* M6 the unclassified *Pseudomonas* like strains 3806/56 and 9631/62 (all three isolated in this laboratory) *Alcalignes faecalis* 415 1347 8769 and *Alcaligenes viscosus* 3233 (all four received from the National Collection of Type Cultures, London with the same designations) The wild type streptomycin sensitivity of the 7 strains was of the same order as that of *Achromobacter* strains (Table 3) and highly streptomycin resistant spontaneous mutants were used for DNA extraction Some conventional characteristics of particular interest are noted in connection with their attempted use as donors of streptomycin resistance (Tables 4 and 6)

The methods of mutant selection and DNA extraction were in accordance with those described (*Bovre* 1964 a)

The continuous DNA exposure technique for comparison and approximate estimation of low transformation compatibilities (*Bövre* 1967) was used in all experiments with mutant DNAs Except for attempts at transfer of haemolysis (methodology described by *Bovre* 1967) streptomycin resistance was the genetic marker Although the mutants were highly resistant transformants were assayed at 50 µg of streptomycin per ml with controls at 10 µg per ml Transformant numbers tabulated are means of two plate counts

Three different experiments with the *Neisseria catarrhalis* Ne 11 recipient and the *Achromobacter lwoffi* 881/57 donor showed that maximum transformant yield was reached with 40 µg or smaller amounts of DNA per ml of the transformation mixture Close to optimal concentration of DNA was therefore considered present in all ordinary experiments

DNA spread on plates 4 5 h *before* the recipient bacteria had practically the same transforming effect as had the same amount spread simultaneously with the reci

parallels to be compared were exposed to streptomycin at exactly the same time also this factor probably was of minor importance for proper deductions from the semiquantitative results It must be emphasized however that different heterolog us transformants may need different periods for phenotypic expression The continuous exposure technique would not permit nondifferential assay of transformants in that case

As in previous transformation studies it was shown that the interactions observed were prevented by DNase (in one case not tested see Results) It must also briefly be mentioned that the interactions were RNase insensitive (tested in several combinations but not all) Repeated DNA extractions and other control measures secured that the results reported were representative Further methodological considerations are made under Results

When transformant DNAs were employed for elucidation of eventual marker heterology the quantitative transformation technique with 15 min DNA exposure was used (*Bovre* 1964 a)

RESULTS

Description of the Achromobacter *Strains*

Inconstancy of the microscopical picture of one and the same strain in different preparations was pronounced Four of the strains are presented in Figs 1–4 The remaining strains were mostly like Figs 2 and 3 but as a rule with slightly smaller dimensions Long filaments could be seen All strains were Gram negative although many of them showed some resistance to decolorization

The colonies had diameters ranging from 1 5 to 2 5 mm They were generally of the low hemispherical or low conical type and were circular, even and glistening The colonies of *Achromobacter lwoffi* had a rather moderate opacity with a clear periphery very little different from the 19116/51 group of oxidase positive rods (*Bovre* 1963 d) The other strains had more opaque colonies No pigmentation was observed on the various media used

No difference was observed as regards growth ability at the two temperatures 32 33° C and 37° C No strain was able to grow under completely anaerobic conditions (hydrogen atmosphere) Stab cultures in semifluid media revealed growth of all strains down to 5 10 mm below the surface

No strain produced indol or hydrogen sulphide Other characteristics of the strains are listed in Table 2 The haemolysis of *Achromobacter haemolysans* presented as wide completely clear zones (diameter 5 6 mm) after incubation for 20 h of human blood agar cultures The haemolysis was bordered by a narrow zone of blood pigment accumulation which gave it a punched out appearance *Achromobacter anitratus* 9 had a little smaller and less clearly delimitated haemolytic zones Growth on Hugh & Leifson's medium was fairly good of all strains on the surface as well as in stab culture Direct comparison with the growth of the 19116/51 group revealed no clear cut difference All *Achromobacter* strains except the two strains [illegible] and 17987 were able to alkalize Simmons Citrate Agar (One strain 881/57) however at the same time seemed completely unable to grow in Koser's

TA[BLE]

Some Characteristics of

Strain	Consistency of colonies	Agglutinability in physiological saline	Growth on Hugh & Leifs medium*
A lwoffi 17985	Soft	—	+
A lwoffi 17987	Mucoid adherent	—	+
A lwoffi 881/57	Soft	—	+
A lwoffi 8858 62	Soft	—	+
A anitratus 8	Soft	—	+
A anitratus 9	Soft	—	+
A haemolysans 742/56	Soft	—	+
A haemolysans 2408/57	Soft	—	+
A haemolysans 2181/60	Soft	—	+
A haemolysans 17988	Slightly friable	+	+

* See text for further description § — = Nitrate apparently not attacked as shown *within 4 days* // — = *No acid production in 20 days on Hugh & Leifson s medium* (: 1 per cent glucose galactose xylose arabinose and 10 per cent lactose respectively

TAB[LE]

Antibiotic Sensitivity of the 10 Achromobacter *Strains (Wild Type) Inhibit*

Strain	Penicillin		Streptomycin	
	Zone in mm	M i c	Zone in mm	M i c
A lwoffi 17985	22	1 4	24	0 6
A lwoffi 17987	22	1 4	24	0 6
A lwoffi 881/57	16	8	26	0 3
A lwoffi 8858/62	<9	>50	30	0 05
A anitratus 8	<9	>50	24	0 6
A anitratus 9	9	>50	27	0
A haemolysans 742/56	9	>50	26	0 3
A haemolysans 2408/57	<9	>50	27	0 2
A haemolysans 2181/60	14	13	24	0 6
A haemolysans 17988	<9	>50	27	0 2

* Method of *Ericsson Högman & Wickman* (1954) M i c values calculated from zone M i c values given as I U/ml for penicillin as μg/ml for the other antibiotics

fluid medium at 32–33° C and 37° C (only at one occasion) The strains 17985 and 17987 grew feebly on all the three citrate media Their growth was directly compared with that of the 19116/51 group and no clear cut difference was observed Although irregularities were observed no strain failed to grow in 1–2 days on more than one of the three media at the two incubation temperatures Difficulties in interpretation of weak growth on citrate media are liable to occur (see *Brooke* 1951 *Klinge* 1959 and the section of Discussion and conclusion) It seems important to repeat the tests preferably on different media Extensive comparisons showed that the cases of weak growth in citrate reported in these studies represent a distinct property not

2
10 Achromobacter *Strains*

Growth on citrate media*	Nitrite production§	Urease activity	Haemolysis*	Gelatin liquefaction*	Acid production from carbohydrates//
+	—	—	—	—	—
+	—	—	—	—	—
++	—	(+)†	—	—	—
++	—	—	—	—	—
++	—	—	—	—	+
++	—	—	+	+	+
++	—	—	+	+	+
++	—	—	+	+	+
++	—	—	+	+	—
++	—	—	+	+	—

means of Zn powder reduction * — = Negative after 20 days of incubation + = Positive culture) + = Acid in 1 2 days on Hugh & Leifson s medium and in fluid media with acid in 20 days in fluid medium with 1 per cent lactose † Weakly pos evident in 2 3 days

3
*Zone Diameters and Approximate Minimum Inhibitory Concentrations (m.i.c.)**

Chloromycetin		Oxytetracycline		Erythromycin	
Zone in mm	M.i.c.	Zone in mm	M.i.c.	Zone in mm	M.i.c.
30	0 6	24	0 6	27	0 8
29	0 8	25	0 4	24	2
30	0 6	24	0 6	25	1
30	0 6	22	1	24	2
19	9	23	0 9	20	6
11	>50	2?	1	21	4
20	7	20	2	20	6
19	9	20	2	21	4
29	0 8	21	2	22	3
19	9	22	1	23	2

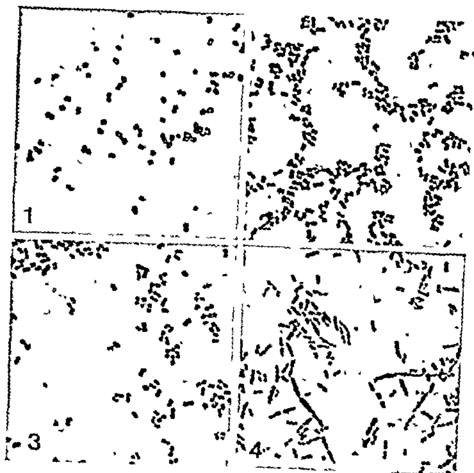
diameters by means of regression equations for each antibiotic (*Ericsson* 1960)

shared by for instance the fastidious *Moraxella nonliquefaciens* (*Bøvre* 1964 b)

The sensitivity to antibiotics of the wild type organisms are presented in Table 3

Transformation Reactions

All strains of *Achromobacter* were tested for competence of being transformed in 15 30 min exposure to own mutant DNA All strains seemed incompetent The strain *Achromobacter lwoffi* 8858 (?) revealed a very slight transformability in continuous DNA exposure Further transformation experiments with *Achromobacter* recipients were not undertaken



Figs 1 4

Fig 1 *Achromobacter lwoffi* 17985 Fig 3 *Achromobacter haemolysans* 17988
Fig 2 *Achromobacter lwoffi* 881/57 Fig 4 *Achromobacter haemolysans* 2408/57
All stained by Gram's method × 1000

Table 4 shows that strain 5873 of the "19116/51" group is transformed to streptomycin resistance by all the *Achromobacter* DNAs when the very sensitive continuous DNA exposure technique is employed However, the transformant counts are very small and little impressive The donors having the greatest similarity to the "19116/51" group in conventional tests (see above) show no peak of activity, and all *Achromobacter* donors are distinctly less effective than the *Moraxella nonliquefaciens* donors in the same experiments The maximum ratio of inter- to intrastrain transformation between the "19116/51" group and *Achromobacter* can be roughly calculated to 1 10 ⁶ (using the activity of *Moraxella nonliquefaciens* 4663 62 as parameter; see Bövre 1967) Motile donors are inactive when applied in parallel

The *Achromobacter* DNAs revealed no transformation compatibility

TABLE 4

Semiquantitative Streptomycin Resistance Transformation of Strain 5873 (of the 19116 51 Group Bovre 1965 d) with Mutant DNAs of Achromobacter *as Compared with the Effects of DNAs of* Moraxella nonliquefaciens *and some Motile Bacteria.*

Experiment no	Donor strains	Resistant colonies per plate* Total	Spontaneous mutants§
1	*A lw* 881/57 *A lw* 8858 62 *A an* 8 *A an* 9 *A haem* 742 56 *A haem* 2181/60 *A haem* 17988	[illegible]1	1
	M nonliq 7784	246	
	M nonliq 4663/62	218	
2	*A an* 9 *A haem* 742/56 *A haem* 2408 57	42 47	2
	A haem 17988	13	
3	*A lw* 17985	30	3
	M nonliq 4663/62	690	
4	*A lw* 17987	22	2
	A haem 2408/57	55	
	M nonl q 7784	427	
	M nonliq 4626 62	367	
	M nonliq 4663 62	506	
	M nonl q 5050 62	783	
	M nonliq 13536 62	321	
	Ps 3806/56 *Alc* 415 *Alc* 3233 *Alc* 8769	2–4	

A lw = *Achromobacter lwoffi* *A an* = *Achromobacter anitratus* *A haem* = *Achromobacter haemolysans* *M nonliq* = *Moraxella nonliquefaciens* *Ps* = *Pseudomonas* like donor (m t le oxidase positive glucose-oxidizing) *Alc* = *Alcaligenes* donors (motile oxidase positive glucose negative)

* Figures can be directly compared only within each experiment

§ Counted on parallel plates with DNase-degraded or no DNA

with another member of the 19116 51 group (strain A1920) *Morax* uefying nonhae ith activities of eventual *Achromobacter* compatibilities with the oxidase positive recipients It is particularly interesting that the nitrate negative strain A1920 is incompatible with the *Achromobacter lwoffi* strains 17985 and 17987

Table 6 shows the activities of all the *Achromobacter* DNAs towards the *Neisseria catarrhalis* Ne 11 recipient The highest compatibility just exceeds the lowest observed between members of the "19116 51 group and the same recipient However the *Achromobacter* activities are distinctly lower than those of *Moraxella nonliquefaciens* The maximum ratio of inter to intrastrain transformation between *Neisseria catarrhalis* Ne 11 and *Achr mobacter* can indirectly be calculated to 7 10 ((using the activity of strain 8292 as parameter B r 1967) Sensitive experiments wer d signed for comparison f th l ast active *Achromobacter* donor with eventu l t vities t n rs of motile

TABLE 5

Attempts at Streptomycin Resistance Transformation of some Oxidase Positive Rods with DNAs of Achromobacter, *with Positive Controls (Continuous DNA Exposure)*

Experiment no	Recipient strain*	Active donor strain*	Resistant colonies per plate: Total	Resistant colonies per plate: Spontaneous mutants§	Donors with no activity in same experiment*
1	A1920	*M nonliq* 4663/62	15	1	*A lw* 17985, *A lw* 17987, *A lw* 8858/62
2	*M nonliq* 7784	8292	35	1	*A lw* 881/57, *A lw* 8858/62, *A haem* 742/56 *A haem* 2408/57, *A haem* 2181/60, *A haem* 17988
3	*M nonliq* 7784	8292	76	3	*A lw* 17985, *A lw* 17987
4	*M bovis* 10900	*N ovis* 199/55	175	2	*A lw* 881/57, *A lw* 8858/62, *A an* 9, *A haem* 2408/57, *A haem* 17988
5	*M bovis* 10900	8292 *N ovis* 199/55	36 255	1	*A lw* 17985, *A lw* 17987
6	(*M lacunata*) A947(1)	8292 *N ovis* 199/55	10 79	2	*A lw* 881/57, *A lw* 8858/62, *A an* 9, *A haem* 2408/57, *A haem* 17988

* *M* = *Moraxella nonliq* = *nonliquefaciens* N = *Neisseria* *A lw* = *Achromobacter lwoffi* *A an* = *Achromobacter anitratus* *A haem* = *Achromobacter haemolysans* Strains with no species designation belong to the 19116/51 group (*Bovre* 1965 d)
(*M lacunata*) See *Bovre* (1965 c)
§ Counted on parallel plates with DNase degraded or no DNA

TABLE 6

Semiquantitative Streptomycin Resistance Transformation of Neisseria catarrhalis *Ne 11 with Mutant DNAs of* Achromobacter *as Compared with the Effects of Other Donors*

Experiment no	Donor strains	Resistant colonies per plate* Total	Resistant colonies per plate* Spontaneous mutants§
1	*A lw* 881/57 *A lw* 8858 62 *A an* 8		4
	A an 9 *A haem* 74?/56 *A haem* 2408'57	56–286	
	A haem 2181/60	50	
	A haem 17988	407	
	8292//	2200†	
	M nonliq 7784	5000*	
	M nonliq 4663/62	5000†	
2	*A lw* 17985 *A lw* 17987	171 224	6
	A haem 17988	253	
	8292//	2500*	
3	*A haem* 17988	457	6
	5873/	309	
	A1920//	650	
4	*A lw* 17987 *A an* 8 *A haem* 17988	352–436	7
	Ps 3806/56	46	
5	*A haem* 2181/60	135	8
	Ps 3806 56	43	
	Ps 9631/6? *Alc* 415 *Alc* 1347 *Alc* 3233		
	Alc 8769 *N meningitidis* M16	<10	
6	*A haem* ?181/60	167	8
	N flavescens 13120	6	

M nonliq = *Moraxella nonliquefaciens* *N* = *Neisseria* *A lw* = *Achromobacter lwoffi* *A an* = *Achromobacter anitratus* *A haem* = *Achromobacter haemolysans* *Ps* = *Pseudomonas* like donors (motile oxidase positive glucose-oxidizing) [illegible] icose negative)

// [illegible]

* [illegible]

§ [illegible]

† Approximate figures

rods *Neisseria meningitidis* and *Neisseria flavescens* Only one of the motile donors showed some activity (Table 6) which was reproducible with different DNA extracts

Some irregularities inherent in the semiquantitative nature of the methodology are observed in Table 6 particularly as regards the relative activities of *Achrom[illegible]acter haemolysans* 17988 and strain 8292 in different experiments

The *Achromobacter* don[illegible] also had som[illegible] effects on the recipients *Neisseria catarrhalis* 4103 [illegible] *Neisseria* [illegible] which were much inferior to the lowest tran[illegible]forming effects [illegible] these recipients of oxidase positive rods [illegible] cocci [illegible] *Neisseria*

TABLE 7

Semiquantitative Streptomycin Resistance Transformation of Neisseria catarrhalis *4103 and* Neisseria ovis *199/55 with Mutant DNAs of* Achromobacter, *Simultaneously Compared with Activites of Oxidase Positive, Immotile Donors*

Experiment no	Recipient strain*	Donor strains*	Resistant colonies per plate Total	Spontaneous mutants§
1	N *catarrhalis* 4103	*A lw* 881/57 *A lw* 8858/62 *A an* 8 *A an* 9 *A haem* 742/56 *A haem* 2408/57 *A haem* 2181/60 *A haem* 17988	9 65	6
		8292	938	
2	N *catarrhalis* 4103	*A lw* 17985 *A lw* 17987 *A lw* 881/57	22–33	10
		8292	264	
3	N *ovis* 199/55	*A lw* 881/57 *A an* 8 *A an* 9 *A haem* 2181/60, *A haem* 17988	32 75	2
		A lw 8858/62	19	
		A haem 742/56	65	
		A haem 2408/57	72	
		M nonliq 7784	6500†	
		M nonliq 4663/62	6500†	
4	N *ovis* 199/55	*A lw* 17985 *A lw* 17987	32 49	1
		A haem 2408/57	35	
5	N *ovis* 199/55	*A haem* 742/56	28	3
		5873	505	
		A1920	466	
		N *catarrhalis* Ne11	2800†	
6	N *ovis* 199/55	*A lw* 8858/62	23	4
		N *flavescens* 13120	3	

* N = *Neisseria* *M* = *Moraxella nonliq* = *nonliquefaciens* *A lw* = *Achromobacter lwoffi* *A an* = *Achromobacter anitratus* *A haem* = *Achromobacter haemolysans*
Strains with no species designation belong to the 19116/51 group (*Bövre* 1965 d)
° Figures can be directly compared only within each experiment
§ Counted on parallel plates without DNA † Approximate figures

flavescens donor, which again was inactive in an experiment where the least active *Achromobacter* donor showed activity (Table 7) The highest compatibility observed between the *Neisseria ovis* recipient and *Achromobacter* can be expressed by the ratio 2 10 ⁻⁶ of inter- to intra strain transformation (roughly estimated from the results of Table 7 using the activity of strain 5873 as parameter see *Bovre* 1967) It must be noted that the interactions between *Neisseria ovis* and *Achromobacter* were not controlled for DNase sensitivity The activity was so low that the assumed transformation could not be reproduced regularly

With the above mentioned exception, the results were generally

reproduced with a satisfactory exactness In one puzzling case, however, distinctly deviating results were obtained with one particular DNA extract of *Achromobacter lwoffi* 17985 The extract had been stored at —20° C at the unusually low concentration of 75 μg per ml for 3 years before use and had an activity towards the recipient strain 5873 (of the "19116.51" group) which was 5–8 times as high as that of several extracts of *Moraxella nonliquefaciens* donors Two freshly prepared extracts of the same mutant had the ordinary low effect on this recipient (Table 4) It may be argued that the higher hetero logous activity observed was due to denaturation by storing at a low concentration That the occurrence of heterologous transformants may depend on the presence of relatively short DNA fragments, has been suggested by *Marmur, Seaman & Levine* (1963) Own experiments with weak shearing forces (repeated vigorous expulsion through a narrow pipette opening) did not indicate that mechanical factors of the usual procedure could have been of importance in the heterologous reactions reported Different degrees of purification are probably un important (*Bovre* 1964 a)

In not tabulated quantitative experiments the strain 5873 and *Neisseria catarrhalis* Ne 11 were exposed to DNAs extracted from transformants elicited in these bacteria by *Achromobacter* donors The only heterologous material of the donors in these second round transformation experiments thus was the part of the genome transferred with the marker in first round transformation Ten transformant DNA extracts were involved and no one was less active than 4 5 10 ¹ of completely homologous transformation Provided that the picked colonies were transformants and not mutants, this result indicates close similarity of the nucleotide sequence of the marker sites in the organisms in question (see *Bövre* 1967)

Transformant assay plates from most of the combinations of first round transformation were replicated to 1000 μg of streptomycin per ml With the exception of a couple of colonies on one occasion all transformants grew at this concentration Some 14 000 of the first round transformants elicited by *Achromobacter* were tested for spontaneous loss of the acquired streptomycin resistance by replica plating to media without streptomycin and with 50 μg of streptomycin per ml No instability was observed

Attempts at transformation of haemolysis from *Achromobacter haemolysans* 17988 to *Neisseria catarrhalis* Ne 11 and from *Achromobacter anitratus* 9, *Achromobacter haemolysans* 74236 and *Achromobacter haemolysans* 17988 to a nonhaemolytic mutant of *Neisseria ovis* (see *Bövre* 1967), all failed

DISCUSSION AND CONCLUSION

As a whole, the *Achromobacter* strains of the present material fulfil the criteria of *Acinetobacter*, as proposed by *Brisou & Prevot* (1954) and *Brisou* (1957).

The strains 17985 and 17987 of *Achromobacter lwoffi* are in conventional tests similar to group 5 or *Achromobacter metalcaligenes* in the classification system of *Mannheim & Stenzel* (1962). The authors listed these organisms as citrate negative (not alkalizing), but it was noted that some of them were able to grow weakly on citrate. It seems that these two strains are similar to those *Moraxella lwoffi* strains examined by *Piechaud, Piechaud & Second* (1951) which grew on citrate media without alkalizing Simmons citrate. The other two *Achromobacter lwoffi* strains of the material seem to coincide with group 4 or *Achromobacter citroalcaligenes* of Mannheim & Stenzel. The remaining strains of *Achromobacter* included are presumably representatives of groups 1, 2 and 3 of the same system (*Achromobacter mucosus*, *Achromobacter haemolyticus* and possibly also *Achromobacter conjunctivae*). Since *Mima polymorpha* (*De Bord* 1939, 1942) and *Moraxella lwoffi* (*Audureau* 1940) probably are the same organisms (*Henriksen* 1963), the *Achromobacter lwoffi* strains of the material may be said also to represent *Mima polymorpha*. For further discussion of designations with relation to the material, *Henriksen* (1963), *Stenzel & Mannheim* (1963) and *Pickett & Manclark* (1965) may be consulted.

It is evident from the study of conventional characteristics that the strains *Achromobacter lwoffi* 17985 and 17987 are virtually indistinguishable from nitrate negative members of the "19116/51" group of oxidase positive rods, except for the negative oxidase reaction. The genetic affinities of these organisms probably constitute main points on which to attack questions of relationship between oxidase positive and oxidase negative organisms.

Thus far, transformation experiments have given no proof or indication of close relationship between the "19116/51" group and oxidase negative rods. The very low frequent interactions observed cannot be overlooked, however, not least because motile donors tested in parallel were inefficient.

The importance of the interactions between the "19116/51" group donors and *Neisseria catarrhalis* may be doubted, since their frequencies are partly overlapped by *Achromobacter*/*Neisseria catarrhalis* interactions. However the absence of activity of other donors (most motile rods, *Neisseria meningitidis* and *Neisseria flavescens*) throws some positive weight on the interactions of both combinations.

The "19116/51" group seems to be clearly closer to the unequivocal moraxellae and *Neisseria ovis* than is *Achromobacter* in these terms. Generally the distinctly inferior transformation compatibilities of this

report as compared with those of the preceding one (*Bovre* 1967) strengthen the belief that the interactions between the unequivocal moraxellae and *Neisseria ovis* have a significant taxonomic meaning. It is also shown that the interactions between the unequivocal moraxellae and *Neisseria catarrhalis* have a relatively outstanding efficiency.

SUMMARY

In semiquantitative streptomycin resistance transformation the activities of 10 *Achromobacter* donors were compared with the activities of selected oxidase positive donors towards various oxidase positive recipients. With few exceptions the *Achromobacter* donors were either inactive or exerted only a small fraction of the lowest efficiency shown by oxidase positive donors.

No indication was provided of close relationship between oxidase positive and oxidase negative rods, although some very infrequent interactions were observed between the 19116/51 group and *Achromobacter*. Between the latter and unequivocal moraxellae no transformation reactivity was found.

Interactions between *Achromobacter* donors and *Neisseria catarrhalis* were partly of the same efficiency as those found between the 19116/51 group and *Neisseria catarrhalis*. Towards a *Neisseria ovis* recipient *Achromobacter* was far less active than the least active donor of the 19116/51 group tested.

The donor capacity of *Achromobacter* was compared with donors of *Alcaligenes* and *Pseudomonas* like organisms towards recipients of the 19116/51 group and of *Neisseria catarrhalis*. With one exception the motile donors were inactive.

Interactions between nonpigmented asaccharolytic *Neisseria* recipients and unequivocal moraxellae were always more frequent than the reactions between the same recipients and *Achromobacter*. The notion that there are relations of taxonomic importance between *Neisseria* and *Moraxella* was therefore strengthened.

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TRANSFORMATION AND DNA BASE COMPOSITION IN TAXONOMY, WITH SPECIAL REFERENCE TO RECENT STUDIES IN *MORAXELLA* AND *NEISSERIA*

By

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Received 25 vi 66

The philosophy and outlines of new categories of approaches to bacterial taxonomy have recently been reviewed and discussed (*Ravin* 1960, 1963, *Bisset* 1962, *Cowan* 1962, 1965, *Floodgate* 1962, *Roper* 1962, *Sneath* 1962, 1964, *Henriksen* 1963 a *Luria* 1963, *Marmur, Falkow & Mandel* 1963, *Pakula* 1963, *Skerman* 1963 *Sokal & Sneath* 1963, *Veron* 1964 and *Colobert* 1965) One important theoretical direction is represented by the numerical analysis of phenotypic expressions By this method all characteristics are given equal weight in the construction of classification groups Another main trend is the use of genetic compatibility and homology of informational macromolecules (nucleic acids and proteins) Genetic transformation and overall DNA base ratio determination constitute important parts of the latter approach

Although transformation and DNA base determination both have theoretical utility in taxonomy, their evaluation is to a large extent empirical Previously obtained data are therefore of great importance for the subsequent discussion of own studies in *Moraxella, Neisseria* and some other organisms

PREVIOUS APPLICATION OF HETEROLOGOUS TRANSFORMATION

The present knowledge of transformation as a genetic phenomenon is mainly based on studies in *Haemophilus*, *Streptococceae*, *Bacillus* and *Neisseria* (see reviews by *Ravin* 1961, *Marmur Falkow & Mandel* 1963, *Hayes* 1964, *Schaeffer* 1964) The majority of th studies concern intra strain or autologous transformation However there are also illustrating examples of described r activity between assumed species or types, which will be considered below

General Principles

A feature reasonably common to "interspecific" transformation reactions is their low frequency when compared with the "intraspecific" kind. However, in some instances reactions exceeding parallel intrastrain reactivity have been observed (*Catlin & Cunningham* 1961, *Marmur, Seaman & Levine* 1963). Such recordings might have been due to slightly different markers in the two parallels, so that transformant selection has been differential.

The ratio of inter- to intrastrain transformation is generally considered to be independent of such variables as DNA concentration, DNA exposure time and time allowed for phenotypic expression (*Schaeffer* 1958). No case of infrequent (but positive) intertransformation has been shown to be due to less effective penetration of heterologous than homologous DNA. Nondifferential penetration of DNA generally seems to have a wider distribution than the intertransformation phenomenon (see *Bovre* 1964 a and *Schaffer* 1964 for discussion and references).

According to the imperfect-pairing hypothesis of *Schaeffer* (1958) the lower inter- than intrastrain transformation frequencies are due to incomplete homologies of the genomes in question, with resulting imperfect pairing of recipient and donor DNAs. As also emphasized by *Schaeffer* (1964), the imperfect pairing hypothesis would be complicated by the eventual presence in transformation of mechanisms similar to host-induced variation of bacteriophage and of bacterial DNA in transduction, conjugation and episomal transfer (*Arber & Dussoix* 1962, *Dussoix & Arber* 1962, *Arber* 1964, *Arber & Morse* 1965, *Wood* 1966). The operation of such factors would not necessarily invalidate transformation as a measure of relatedness, however.

Transformation in Haemophilus

Schaeffer & Ritz (1955) and *Alexander & Leidy* (1955) provided for the first time evidence of transformation between species of *Haemophilus*. *Leidy, Hahn & Alexander* (1956) reported ratios of inter to intrastrain streptomycin resistance transformation ranging from $2 \cdot 10^{-3}$ to $2 \cdot 10^{-2}$ (in the present author's own terminology) between *Haemophilus influenzae* and *Haemophilus parainfluenzae*. Between the former and *Haemophilus suis* (not requiring X factor, however) the ratios ranged from $1 \cdot 10^{-4}$ to $4 \cdot 10^{-4}$. These values were verified with a very striking accuracy by *Schaeffer* (1956, 1958). *Leidy, Hahn & Alexander* (1959) found corresponding ratios ranging from 1/5 to 1 between *Haemophilus influenzae* and *Haemophilus aegyptius*.

On the basis of their transformation results Leidy, Hahn & Alexander (1956) concluded that *Haemophilus influenzae* is more closely related to *Haemophilus parainfluenzae* than to the strain called *Haemophilus suis*. Further evidence of genetic homology between *Haemophilus*

influenzae and *Haemophilus aegyptius* was presented by *Leidy, Jaffee & Alexander* (1965) *Haemophilus influenzae* and *Haemophilus aegyptius* are morphologically very similar and it may be questioned even on the basis of conventional criteria whether they deserve species distinction

The transformation compatibilities of *Haemophilus influenzae, Haemophilus parainfluenzae* and the type of *Haemophilus suis* used above are reflected in the guanine + cytosine (G + C) contents of their DNAs 38 39 and 41 per cent respectively (*Schaeffer, Edgar & Rolfe* 1960) *Marmur, Falkow & Mandel* (review 1963) reported G + C values from 38 to 40 per cent of all the *Haemophilus* species mentioned including *Haemophilus aegyptius*

Transformation in Streptococceae

Bracco et al (1957) *Pakula Fluder, Hulanicka & Walczak* (1958) and *Pakula Hulanicka & Walczak* (1958 1959) reported transformation reactions between species of *Streptococcus* and partly also between *Diplococcus pneumoniae* and streptococci Quantitative results were given by *Pakula* (1961 1963) Calculations from his figures show ratios of inter to intrastrain streptomycin resistance transformation of the order 1 between *Streptococcus sanguis* and a strain of group H streptococci in reciprocal tests Between these strains and *Diplococcus pneumoniae* ratios ranged from 8 10^{-3} to 6 10 [illegible], also in reciprocal tests With *Streptococcus mitis* donors the *Diplococcus pneumoniae* recipient revealed ratios from 2 10^{-3} to 1 10^{-1} Between the same recipient and donors of *Streptococcus salivarius* group A and group C streptococci ratios of the order 4 10^{-6} to 9 10^{-3} were found Between the group H and *Streptococcus sanguis* recipients and donors of *Streptococcus mitis* ratios were of the order 1 10 [illegible] to 6 10 [illegible] and between the same recipients and *Streptococcus salivarius*, group A and group C donors ratios ranged from 2 10^{-4} to 9 10^{-3} With a *Streptococcus faecalis* donor the ratios ranged from 4 10 [illegible] to 8 10^{-4}

Results of streptomycin resistance transformation performed by *Perry & Slade* (1962 1964) in *Streptococcus* were in close agreement with the studies of Pakula and collaborators Further evidence of genetic homology between group H streptococci and *Diplococcus pneumoniae* was provided by *Ravin* (1963) and *Ravin & De Sa* (1964)

On the basis of his own results *Pakula* (1963) concluded that *Diplococcus pneumoniae* is closer in these terms to some streptococci than many streptococci are to each other and emphasized the agreement with the classification of Topley and Wilson *Wilson & Miles* 1955 and also 1964) where pneumococci and streptococci are not separated as distinct genera He also emphasized that the genetic evidence of species identity of *Streptococcus sanguis* and group H streptococci corroborates physiological and immunological data The results with

Streptococcus faecalis DNA were taken to indicate its also otherwise presumed distant relationship to other streptococci

Overall DNA base ratios of *Diplococcus pneumoniae*, *Streptococcus salivarius*, *Streptococcus sanguis*, *Streptococcus mitidans* and *Streptococcus pyogenes* are all situated at 38–40 per cent G + C. *Streptococcus faecalis* has G + C values in the interval 34–36 per cent (*Marmur, Falkow & Mandel* 1963)

Transformation in Bacillus

Dubnau et al (1965) transformed *Bacillus subtilis* (43 per cent G + C) to streptomycin resistance with DNAs of *Bacillus niger* (43 per cent G + C), *Bacillus pumilis* (40–41 per cent G + C) and *Bacillus licheniformis* (45–46 per cent G + C). The ratios of inter- to intrastrain transformation were $5.4 \cdot 10^{-3}$, $2.3 \cdot 10^{-2}$ and $4 \cdot 10^{-3}$ respectively. Transformation with biochemical markers was much inferior or negative in the same study, which is in accordance with previously obtained results in *Bacillus* (*Marmur, Seaman & Levine* 1963)

Transformation in Neisseria

Catlin (1960, 1961) and *Catlin & Cunningham* (1961) examined *Neisseria meningitidis*, *Neisseria flava*, *Neisseria perflava*, *Neisseria subflava*, *Neisseria sicca* and *Neisseria flavescens* in quantitative streptomycin resistance transformation. Between all these organisms ratios of inter- to intrastrain transformation were $1 \cdot 10^{-2}$ or higher. Between *Neisseria flava*, *Neisseria perflava* and *Neisseria subflava* ratios were higher than 1/10 (in both directions and all possible combinations). On the other hand a highly competent *Neisseria catarrhalis* recipient underwent virtually no transformation in response to DNAs from the above mentioned organisms (ratios less than $2 \cdot 10^{-6}$). In striking correlation with this were results of DNA base determinations (*Catlin & Cunningham* 1961), which revealed approximately 41 per cent G + C for *Neisseria catarrhalis* and approximately 50 per cent G + C for the other organisms. The authors stated that the inclusion of *catarrhalis* strains in genus *Neisseria* appears illogical from the evolutionary point of view, whereas the other six organisms mentioned might be regarded as members of a single group (group I). The authors also emphasized that the combination of *Neisseria flava*, *Neisseria perflava* and *Neisseria subflava* into a single species, as suggested by *Wilson & Miles* (1955 and also 1964), is in harmony with evidence from the transformation experiments

DNA BASE COMPOSITION AND ITS RELATION TO TAXONOMY

Among bacteria the overall base composition of DNA, as expressed in mole per cent guanine + cytosine (G + C) of total bases, varies from 25 to 75 approximately (*Sueoka* 1961). Within a bacterial strain the

G + C content shows a unimodal distribution and the range of heterogeneity between DNA molecules is relatively narrow (*Rolfe & Meselson* 1959, *Sueoka* 1961). Within molecules, however, the range of local composition seems rather broad (*Guild* 1963), contrary to earlier belief (*Sueoka* 1961).

Generally, similar overall DNA base composition is considered a necessary requirement for extensive base sequence homology, as reflected in genetic compatibility and integration (*Lanni* 1960, *Luria* 1963, *Marmur, Falkow & Mandel* 1963). However, transformation may occur between microbes which differ in G + C content by as much as 3–4 per cent (*Catlin* 1964, *Catlin & Cunningham* 1964 a, *Dubnau et al* 1965). Some instances of genetic transfer between organisms of widely different G + C contents have been reviewed by *Marmur, Falkow & Mandel* (1963). In these cases the genetic information is considered to be mediated by episomes which are not integrated in the resident chromosome. *Falkow et al* (1961) and *Marmur et al* (1961) have presented very clear evidence of such transfer between *Salmonella typhosa* and *Escherichia coli* (both 50 per cent G + C) on one side and *Serratia marcescens* (58 per cent G + C) on the other. However, there probably also exists a limit to the range of base composition compatible with episomal infection. It has been suggested that this mode of genetic transfer is indicative of large taxonomic groupings, such as a family (*Marmur, Falkow & Mandel* 1963, *Mandel* 1966).

On the other hand, identical or closely similar DNA base ratios are evidently not sufficient for genetic interaction to occur, as emphasized for transformation in *Bacillus* (*Marmur, Seaman & Levine* 1963). Other illustrating examples are some combinations of enterobacteria, in which inferior efficiency in transduction and conjugation reflects only partial base sequence homology among organisms of similar overall DNA base composition (*Schildkraut, Marmur & Doty* 1961, *Marmur, Falkow & Mandel* 1963).

A large body of information has been made available on overall DNA base composition of bacteria since *Lee, Wahl & Barbu* (1956) presented the first analysis of its importance as a taxonomic aid. Reports (the first four relatively extensive reviews) which together contain most of the data known are those of *Sueoka* (1961), *Marmur & Doty* (1962), *Schildkraut, Marmur & Doty* (1962), *Marmur, Falkow & Mandel* (1963), *Catlin & Cunningham* (1961, 1964 a, b), *Sebald & Véron* (1963), *Colwell & Mandel* (1964), *De Ley & Rassel* (1965), *Mandel & Leadbetter* (1965), *Seese, Jeffries & Mandel* (1965), *De Ley et al* (1966), *Gasser & Sebald* (1966), *LaMacchia & Pelczar* (1966) and *Mandel* (1966).

In some cases good correlation has been found between overall DNA base composition and groups constructed by means of numerical analysis (*Colwell & Mandel* 1964, *Colwell, Mandel & Gochnauer* 1964, *De Ley & Rassel* 1965, *Silvestri & Hill* 1965). The majority of species

presently organized in particular genera seem to have DNA base compositions of quite similar order, and differences in these terms are often clear cut between higher taxa of classical taxonomy There are exceptions, however In some cases heterogeneity is so great within a taxon that it calls for reconsideration of the classification system In other cases identity or close similarity in DNA base composition is found among microbes of great phenotypic diversity, as between *Streptococceae, Proteus vulgaris, Haemophilus influenzae, Bacillus megaterium* and *Achromobacter* strains Determination of overall DNA base composition therefore has its limitations in taxonomy when used alone

OWN STUDIES IN *MORAXELLA NEISSERIA* AND SOME OTHER ORGANISMS

The transformation studies to be commented upon have been published elsewhere (*Bovre* 1964 a, b, 1965 a b c d, 1967 a, b) The strains employed and the main results are listed in Tables 1, 3 and 4

Results of DNA base determinations, performed by Dr W Szybalski* with the CsCl buoyant density method on DNA extracts prepared by the author, are given in Tables 2 and 3 The results were generally not available before the respective transformation experiments had been finished The methodology of the DNA base determinations will be included in a separate report (*Bovre Szybalski & Frandt* 1966)

The Marker System and its Implications

The streptomycin system seems to be essentially similar in the strains involved, at least as regards interacting strains A satisfactory marker uniformity is indicated by high degrees of sensitivity of all wild type organisms towards this antibiotic, by high degree of streptomycin resistance in all spontaneous one step mutants used as donors and by wide distribution of transformation with this marker

Numerous experiments with integration of marker of heterologous origin in various donors have revealed no marker associated heterologies capable of reducing a first degree compatibility (whenever the term marker is used in this section, streptomycin resistance is meant if not otherwise stated) The incompatibility of *Neisseria cinerea* and *Neisseria flavescens* with the other oxidase positive organisms is paralleled by obvious gaps in terms of G + C content (Table 2), and the importance of discussion of marker associated heterologies is reduced in these cases

It has been shown that heterologous transformation reactions are positively influenced by previous integration (in transformation) of homologous marker in the heterologous donor In that manner very low frequent first round transformation has been raised to an inter

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mediate or higher level in second round transformation It may be suggested that homology restricted to the marker site can allow for only extremely low frequent transformation if any To what extent more extensive but still particularly marker associated homologies may affect transformation frequency in a positive way without artificial preintegration is undecided However, the most low frequent reactions will be basis of very limited taxonomic conclusions In transformation of intermediate efficiency DNA base composition and transformation results with another marker (haemolysis) indicate widespread homologies (see below)

Some of the points emphasized above are based on the assumption that Schaeffer's pairing hypothesis is valid (see previous discussion) and not complicated by differential expression of intrastrain and interstrain transformants It has repeatedly been observed that mutants at the streptomycin locus may have a pleiotropic nature with one or more metabolic requirements (*Gorini & Beckwith* 1966 review) In *Rhizobium*, nutritionally restricted streptomycin resistant mutants were observed (*R Balassa* 1954 *G Balassa* 1963) It appeared that the development of heterologous transformants depended on the same factors as those required for development of mutants of the donors in question (not the recipients) It is therefore important to note that in the present studies the donor mutants were selected in the same manner and on the same media as was the case with transformants

Although streptomycin resistance transformation may be differentially depressed even between closely related strains the deviations observed in these cases have been relatively small (*Green* 1959 *Roiheim* 1962) Lowering of the transformant selection concentration of streptomycin may be of importance (*Bryan* 1961 *Pakula & Hulanicka Banikowska* 1961 *Rotheim & Ravin* 1961 *Catlin & Cunningham* 1964 b *Bovre* 1965 c 1967 a) eventually uncovering what might be called true transformation frequency

Mutations at the streptomycin locus correspond to structural changes in ribosomes (*Spotts & Stanier* 1961 *Cox White & Flaks* 1964 *Davies* 1964) Ribosomes play an important part in the process of translation of the genetic message and streptomycin is considered to exert its effect by interfering with translation (*Gorini & Kataja* 1964 *Davies Gilbert & Gorini* 1964 *Gorini & Beckwith* 1966 review) It may therefore be argued that the streptomycin system has intimate relation to a widespread function so that integration and expression barriers are less likely to occur with streptomycin resistance markers as compared with some other markers *Ravin & De Sa* (1964) emphasized the widespread homology of the streptomycin locus among the streptococci pneumococci and stated that mutual recognition of this locus persists between species even as ability to transform each other declines *Dubnau et al* (1965) presented results of interspecific transformation in *Bacillus* which suggest the existence of a core of stable genetic

presently organized in particular genera seem to have DNA base compositions of quite similar order and differences in these terms are often clear cut between higher taxa of classical taxonomy. There are exceptions however. In some cases heterogeneity is so great within a taxon that it calls for reconsideration of the classification system. In other cases identity or close similarity in DNA base composition is found among microbes of great phenotypic diversity, as between *Streptococceae*, *Proteus vulgaris*, *Haemophilus influenzae*, *Bacillus megaterium* and *Achromobacter* strains. Determination of overall DNA base composition therefore has its limitations in taxonomy when used alone.

OWN STUDIES IN *MORAXELLA*, *NEISSERIA* AND SOME OTHER ORGANISMS

The transformation studies to be commented upon have been published elsewhere (*Bøvre* 1964 a, b, 1965 a, b, c, d, 1967 a, b). The strains employed and the main results are listed in Tables 1, 3 and 4.

Results of DNA base determinations, performed by Dr. W. Szybalski* with the CsCl buoyant density method on DNA extracts prepared by the author, are given in Tables 2 and 3. The results were generally not available before the respective transformation experiments had been finished. The methodology of the DNA base determinations will be included in a separate report (*Bøvre, Szybalski & Fiandt* 1966).

The Marker System and its Implications

The streptomycin system seems to be essentially similar in the strains involved, at least as regards interacting strains. A satisfactory marker uniformity is indicated by high degrees of sensitivity of all wild type organisms towards this antibiotic, by high degree of streptomycin resistance in all spontaneous one step mutants used as donors and by wide distribution of transformation with this marker.

Numerous experiments with integration of marker of heterologous origin in various donors have revealed no marker-associated heterologies capable of reducing a first degree compatibility (whenever the term marker is used in this section, streptomycin resistance is meant if not otherwise stated). The incompatibility of *Neisseria cinerea* and *Neisseria flavescens* with the other oxidase positive organisms is paralleled by obvious gaps in terms of G + C content (Table 2), and the importance of discussion of marker associated heterologies is reduced in these cases.

It has been shown that heterologous transformation reactions are positively influenced by previous integration (in transformation) of homologous marker in the heterologous donor. In that manner very low frequent first round transformation has been raised to an inter

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mediate or higher level in second round transformation It may be suggested that homology restricted to the marker site can allow for only extremely low frequent transformation if any To what extent more extensive but still particularly marker associated homologies may affect transformation frequency in a positive way without artificial preintegration is undecided However the most low frequent reactions will be basis of very limited taxonomic conclusions In transformation of intermediate efficiency DNA base composition and transformation results with another marker (haemolysis) indicate widespread homologies (see below)

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material, relatively resistant to evolutionary change Included in this core is the streptomycin locus Ratios of inter- to intrastrain transformation with several biochemical markers were below 5 10⁻³ even between strains of the same species, whereas corresponding ratios with the streptomycin resistance marker kept easily measurable between species, falling off gradually with deviation of overall DNA base composition

In contrast to the apparently uniform distribution of genetic fundaments and phenotypic expression of streptomycin resistance, stands the transformation of haemolysis (*Bovre* 1967 a, b) This natural marker is evidently not of the ubiquitary character wanted for the purpose of the present studies

Ravin (1963) suggested that some instances of interspecific transformation in the past may have been due to infection by an unintegrated episome (for reviews on episomes, see *Jacob, Schaeffer & Wollman* 1960, *Campbell* 1962, *Driskell-Zamenhof* 1964, *Hayes* 1964) Discussion of such a mechanism in the present system does not seem relevant, however, since the DNA extraction procedure employed (*Bovre* 1964 a) with great probability is incompatible with persistence of an eventual infectious episome (*Jyssum* 1966) The unknown genetic determinant of haemolysis might nevertheless have an extrachromosomal location although the stability of the transferred character seemed great

General Deductions from the Transformation Data

Streptomycin resistance transformation appears to be a valuable aid in exact ordering and control of identity of individual strains A few examples will be mentioned

It now seems that *Neisseria catarrhalis* usually is nitrate reducing (*Bovre* 1965 b) Nitrate negative, asaccharolytic neisseriae are not easily classified (*Neisseria cinerea* and *Neisseria catarrhalis* 4103, see below) The nitrate negative strain *Neisseria catarrhalis* 13074/62 (*Bovre* 1965 b) was found to possess a first degree relationship in terms of streptomycin resistance transformation to nitrate positive *Neisseria catarrhalis* organisms It was consequently proposed that the strain was an unusual variety of *Neisseria catarrhalis*, but not representing a distinct taxonomic entity DNA base ratios available at a later stage corroborated the transformation result (Tables 1 and 2)

During the studies a nonhaemolytic variety of *Neisseria ovis* was isolated A nonhaemolytic *Neisseria ovis* is practically indistinguishable from *Neisseria catarrhalis* in the conventional tests used (*Bovre* 1965 b) Quantitative transformation with two markers revealed the true nature of the variety (*Bovre* 1967 a)

The strains 19116/51 and 752/52 both deviated from other assumed *Moraxella nonliquefaciens* strains in conventional tests (*Bovre* 1964 b) In transformation the deviation was very distinct and once more the DNA base ratios were corroborative (Tables 1 and 2)

9*

TABLE 1

Strains Studied by means of Streptomycin Resistance Transformation. Ratios of Inter- to Intrastrain Transformation

Provisional species or group designation*	Strain designations (I)	Strain designations (II)	Ratios of inter- to intrastrain transformation: Among (I)	Ratios of inter- to intrastrain transformation: Between (I) and (II)
Moraxella nonliquefaciens	7784, 672/58, 270 CO, 2770 CO, 3828 CO, 826/61, 836/61, 159/62, 178/62, 1962/62, 4235/62, 4378/62, 4626/62, 4663/62, 4863/62, 5050/62, 5058/62, 13015/62, 13385/62, 13536/62	752/52 19116/51§	$3.4 \cdot 10^{-1}$ — $9.9 \cdot 10^{-1}$	$<2.1 \cdot 10^{-5}$ $<2.1 \cdot 10^{-5}$
Moraxella bovis	8561, 9425, 9426, 10900		$5.3 \cdot 10^{-1}$ — $1.1 \cdot 10^{0}$	
Serum liquefying nonhaemolytic moraxellae	7911, 9833, 9985, E1, I 1, A947(1), 11748		$1.0 \cdot 10^{-1}$ — $9.6 \cdot 10^{-1}$	
"19116/51" group of oxidase positive rods	5718, 5873, 5893, 8134, 8292, 9893, 10973, 19116/51, AC08, A1920		$3.2 \cdot 10^{-1}$ — $1.0 \cdot 10^{0}$	
Neisseria catarrhalis	Ne11, 8176, 12910/62, 13016/62, 13074/62, 13135/62, 13430/62, 2833/63, 2982/63, 2993/63, 7889/63, 1163/64, 1179/64, 2424/64	4103	$3.0 \cdot 10^{-1}$ — $9.4 \cdot 10^{-1}$	$5.4 \cdot 10^{-4}$ — $4.6 \cdot 10^{-3}$
Neisseria caviae		10293, 14659	Incompetent†	
Neisseria ovis	199/55, 17/59, 917/60		$9.1 \cdot 10^{-1}$ — $9.5 \cdot 10^{-1}$	
Neisseria cinerea		165/61, 159/62	Incompetent†	
Neisseria flavescens	8263, 13115, 13120		$4 \cdot 10^{-1}$°	
Achromobacter//		17985, 17987, 881/57, 8858/62, 8 9, 742/56, 2108/57, 2181/60, 17988	Incompetent†	

* The conventional characteristics of all strains have been reported (*Bøvre* 1964 b, 1965 a, b, c, d, 1967 b). § Representative of the "19116/51" group. † No transformants in 15 min exposure to own mutant DNA. ° Semiquantitative estimate. // See Table 2.

TABLE 2

Mean Guanine + Cytosine (G + C) Content of DNA Extracted from Oxidase Positive and Oxidase Negative Organisms (Part of the Strains of Table 1).

Provisional species or group designation	Strains examined	CsCl buoyant density (g/cm³)	Mole per cent G + C
Moraxella nonliquefaciens	7784, 2770/60, 3828/60, 826/61,		
	836/61, 4378/62, 4863/62,		
	5058/62, 13536/62	1 701	41
	178/62	1 700	40
	5050/62, 13385/62	1 7015	41 5
	4663/62	1 702	42
	752/52*, 19116/51*	1.7035	43 5
Moraxella bovis	8561, 9425, 10900	1 7025	42 5
	9426	1 703	43
Serum-liquefying, nonhaemolytic moraxellae	9833, 9985, Γ1, L1	1 703	43
	7911	1 7015	41 5
	11748	1 702	42
	A947(1)	1 7025	42 5
"19116/51" group of oxidase positive rods	5718, 5873, 8134, 19116/51,		
	A608, A1920	1 7035	43 5
	5893 8292, 9893	1 703	43
Neisseria catarrhalis	Ne11 12910/62 13016/62, 13074/62,		
	13135/62, 13430/62, 2833/63,		
	2982/63 7889/63 1163/64, 1179/64,		
	2424/64	1 701	41
	8176	1 702	42
	4103*	1 7025	42 5
Neisseria caviae	10293, 14659	1 7045	44 5
Neisseria ovis	37/59, 917/60	1 7045	44 5
	199/55	1 705	45
Neisseria cinerea	165/61, 159/62	1 709	49
Neisseria flavescens	8263	1 7065	46 5
	13115	1 707	47
	13120	1 7075	47 5
Achromobacter lwoffi	17985	1 703	43
	17987	1 7035	43 5
	881/57	1 7015	41 5
	8858/62	1 698	38
Achromobacter anitratus	B 9	1 6995	39 5
Achromobacter haemolysans	742/56	1 705	45
	2408/57	1 7015	41 5
	2181/60	1 6995	39 5
	17938	1 701	41

* Deviating in terms of transformation (see Table 1)

TABLE 3

Range of G + C Content of Entities Considered equivalent to Species on the Basis of Streptomycin Resistance Transformation (Ratios of Inter to Intrastrain Transformation 1 0 10 1 or Higher see Table 1) and Interspecies Streptomycin Resistance Transformation within Arbitrary Groups

Arbitrary group	Assumed entity equivalent to a species	Range of per cent G + C	Range of ratios of inter to intrastrain transformation between entities of the same group
A	1 *M nonliquefaciens** 2 *M bovis* 3 Serum liquefying nonhaemolytic moraxellae 1 *M nonliquefaciens**	40–42 42 5–43 41 5 43 40–42	1 5 10^{3} – 7 5 10^{3} 1 2 10^{-3} – 1 8 10^{2} 1 0 10^{3} – 5 2 10^{3}
B	19116/51 group	43 43 5	
C	1 *N caviae* 2 *N ovis*	44 5 44 5 45	4 9 10^{-4} – 5 6 10^{4}*
D	1 *N catarrhalis* (typical) 2 *N catarrhalis* 4103	41 42 42 5	5 4 10^{-4} – 4 6 10^{3}
F	1 *N cinerea* 2 *N flavescens*	49 46 5–47 5	5 10^{2} 6 10^{-2}§†

M = *Moraxella* *N* = *Neisseria*

* The deviating strain 752/52 excluded Strain 19116/51 also excluded and moved to the 19116/51 group

§ One way transformation reactions

† Semiquantitative estimates expressed in quantitative terms

During the further study of the 19116/51' group (*Bovre* 1965 d) it was found that nitrate reduction varied within the genetically homogeneous group and therefore could not be used for distinction from the presumably regularly nitrate reducing *Moraxella nonliquefaciens* It was shown that other conventional characteristics followed the transformation pattern and later also DNA base ratios (Table 2)

Some of these examples show that weighting of conventional characteristics in a group of bacteria may be put on a sound basis by the application of quantitative transformation An easily performed high frequency transformation experiment may also be employed directly in some cases of problematic diagnosis when a system of genetically arranged microbes is availabl

A first degree transformat n compatibility (ratios of inter to intra strain transformation to str tomycin resistance ranging from 1/3 to 1) may be taken as an indicat r of widely distributed homologies of the genomes in question (see *B re* 1964 b 1965 b d and Tables 1 and 2) Findings by other investigators (see previous discussion) have even shown that if reciprocal streptomycin resist ransformation ratios are 1 10 or higher the str ins in question h ular affinities also

by other means, and species identity has been discussed also on the basis of other criteria alone

For several reasons an entity with compatibility ratios from 1/10 to 1 in streptomycin resistance transformation (preferably reciprocal) may be suggested as a base-line above the strain concept in bacterial classification Delimitation of a species will be based on this genetically defined entity in the following discussion Within such a species concept variation of G + C content is 2 per cent at maximum (Table 3)

On the basis of the transformation studies as a whole one is forced to look at the reactions between *Neisseria cinerea* and *Neisseria flavescens* and also between the fastidious *Moraxella nonliquefaciens* (*Bovre* 1964 b), *Moraxella bovis* and the serum-liquefying, nonhaemolytic moraxellae as indicating particularly close relationships between species (Table 3) The affinities of the two former are comparable to those found between *Neisseria flavescens* and the saccharolytic neisseriae (*Catlin & Cunningham* 1961), and the compatibilities between the moraxellae may be compared with the reactions between some of the closest related *Streptococceae* and between *Haemophilus influenzae and Haemophilus parainfluenzae* (*see previous section of this paper*) Some other interactions deserve special consideration, not least because they partly point to the significance of the compatibilities discussed above The reactions between ordinary *Neisseria catarrhalis* strains and the nitrate negative *Neisseria catarrhalis* 4103 (Bovre 1965 b) are of a similar order or slightly less pronounced than those between moraxellae *Neisseria caviae* and *Neisseria ovis* are a little more distant from each other than are the moraxellae in the same terms Nevertheless they are very similar in conventional terms(*Bovre* 1965 b) and their overall base compositions are identical

The only other species combination which reveals ratios of compatibility higher than the *Neisseria caviae/Neisseria ovis* ratios is the combination *Moraxella bovis/Neisseria ovis* (Table 4) Important is here the apparent existence of DNA regions with more pronounced homologies than the region bearing the streptomycin locus (*Bovre* 1967 a) It is tempting to compare the reactions between *Moraxella bovis* and *Neisseria ovis* with some interactions of *Streptococceae*, for instance (see previous discussion)

Deduction from transformation compability becomes progressively more difficult with the gradually decreasing ratios of inter- to intrastrain transformation The relative magnitude of interactions inferior to 5 10^{-4} may be ordered as follows (Table 4) The affinities of *Moraxella liquefaciens and the fastidious Moraxella nonliquefaciens* to *Neisseria ovis* are partly close to the *Neisseria caviae/Neisseria ovis* interactions The affinities in these terms between *Neisseria catarrhalis* and all three unequivocal *Moraxella* entities, between *Neisseria ovis* and *Neisseria catarrhalis*, between *Neisseria ovis* and the "19116/51" group, between the "19116 51" group and the three unequivocal *Morax-*

TABLE 4

Some Intergroup Reactions in Streptomycin Resistance Transformation *

Recipient Group§	Recipient Entity§ (species)	Recipient Strain	Donor Group§	Donor Entity§ (species)	Ratios of inter- to intrastrain transformation
C	2	199/55	A	2	6.2 10^{-4} – 1.1 10^{-3}
C	2	199 55	A	3	2.4 10^{-4}
C	2	199/55	A	1	7.5 10^{-5} – 1.0 10^{-4}
D	1	Ne11	A	1–2 3	1.8 10^{-5} – 7.4 10^{-5}
C	2	199 55	D	1 2	3.0 10^{-5} – 4.6 10^{-5}
C	2	199 55	B		2.2 10^{-5} – 4.4 10^{-5}
B		5873	A	1 2 3	6 10^{-6}† – 4.0 10^{-5}
D	1	Ne11	B		6 10^{-6}† – 4.0 10^{-5}
D	1	Ne11	C	1–2	9.8 10^{-6} 3.4 10^{-5}
D	1	Ne11	*Achromobacter*		7 10^{-6}†*
C	2	199/55	*Achromobacter*		2 10^{-6}†*
B		5873	*Achromobacter*		1 10^{-6}†*
A			*Achromobacter*		n t
A			E	2	n t
B			F	2	n t
C			F	1–2	n t
D			F	1–2	n t

* Selected experiments generally one way in the most effective transformation direction

§ See Table 3 *Achromobacter* added, see Table 2

† Semiquantitative estimates expressed in quantitative terms

* Maximum ratio observed employing all 10 donors

n t = Transformants not observed in semiquantitative experiments of very high sensitivity

ella entities and between *Neisseria catarrhalis* and the "19116 51" group are rather similar and lower, but still clearly observable The relation to *Neisseria catarrhalis* of *Neisseria caviae* is of the same order as that of *Neisseria ovis* Typical *Neisseria catarrhalis* has the same relation to *Neisseria ovis* as the deviating strain 4103 *Achromobacter* strains have affinities to *Neisseria catarrhalis* which generally are distinctly lower than the ones mentioned above but which also partly overlap the activity of the "19116 51" group on the same recipient in simultaneous experiments (*Bövre* 1967 b) There is a slight activity of *Achromobacter* donors on the *Neisseria ovis* recipient, which generally is doubtful (*Bovre* 1967 b) The activity of *Achromobacter* on a recipient from the "19116 51" group is also very slight The latter activity is distinctly lower than that exerted by unequivocal moraxellae on the same recipient (but see *Bövre* 1967 b) No activity has been observed on *Moraxella* of *Achromobacter* donors, and no interaction of *Neisseria flavescens* with other oxidase positive recipients under the most sensitive test conditions

That a motile, *Pseudomonas* like donor was slightly active in transformation of *Neisseria catarrhalis* (*Bovre* 1967 b) is confusing However, this reaction was distinctly less frequent than any other positive reaction observed Only the future may show the relevance of

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Deduction from transformation compability becomes progressively more difficult with the gradually decreasing ratios of inter- to intrastrain transformation The relative magnitude of interactions inferior to $5 \cdot 10^{-4}$ may be ordered as follows (Table 4) The affinities of *Moraxella liquefaciens* and the fastidious *Moraxella nonliquefaciens* to *Neisseria ovis* are partly close to the *Neisseria caviae*/*Neisseria ovis* interactions The affinities in these terms between *Neisseria catarrhalis* and all three unequivocal *Moraxella* entities, between *Neisseria ovis* and *Neisseria catarrhalis*, between *Neisseria ovis* and the "19116 51" group, between the "19116 51" group and the three unequivocal *Morax-*

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C	2	19955	A	3		$2.4 \cdot 10^{-4}$
C	2	19955	A	1	$7.5 \cdot 10^{-5}$	$1.0 \cdot 10^{-4}$
D	1	Ne11	A	1 2 3	$1.8 \cdot 10^{-5}$	$7.4 \cdot 10^{-5}$
C	2	19955	D	1 2	$3.0 \cdot 10^{-5}$	$4.6 \cdot 10^{-5}$
C	2	19955	B		$2.2 \cdot 10^{-5}$ –	$4.4 \cdot 10^{-5}$
B		5873	A	1 2 3	$6 \cdot 10^{-6}$‡	$4.0 \cdot 10^{-5}$
D	1	Ne11	B		$6 \cdot 10^{-6}$†	$4.0 \cdot 10$
D	1	Ne11	C	1 2	$9.8 \cdot 10^{-6}$	$3.4 \cdot 10^{-5}$
D	1	Ne11	*Achromobacter*			$7 \cdot 10^{-6}$†*
C	2	19955	*Achromobacter*			$2 \cdot 10^{-6}$†*
B		5873	*Achromobacter*			$1 \cdot 10^{-6}$†*
A			*Achromobacter*		n.t.	
A			E	2	n.t.	
B			E	2	n.t.	
C			F	1 2	n.t.	
D			E	1 2	n.t.	

Selected experiments, generally one way in the most effective transformation direction

§ See Table 3. *Achromobacter* added, see Table 2.

‡ Semiquantitative estimates expressed in quantitative terms

* Maximum ratio observed employing all 10 donors

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That a motile *Pseudomonas*-like donor was slightly active in transformation of *Neisseria catarrhalis* (*Bøvre* 1967 b) is confusing. However, this reaction was distinctly less frequent than any other positive reaction observed. Only the future may show the relevance of

this observation In any case, the many non reacting donors of *Alcaligenes Pseudomonas*, *Neisseria meningitidis* *Neisseria flavescens* etc (*Bovre* 1967 a, b) show that there are zero endpoints of the distribution of streptomycin resistance transformation in the vicinity of *Moraxella* even when the most sensitive methods are used

The species concept proposed above should not be considered as a genospecies but as a genetically defined taxonomic species If a genospecies is taken to indicate a group of organisms between which genetic transfer is possible (*Ravin* 1960) it now seems that such a group may be very wide

Transformation Compatibility and DNA Base Composition Applied to Some Actual Taxonomic Questions

Affinities between oxidase positive, immotile rods The fastidious *Moraxella nonliquefaciens* (*Bovre* 1964 b) *Moraxella bovis* (*Bovre* 1965 a) and the serum liquefying nonhaemolytic moraxellae (*Bovre* 1965 c) may be said to represent three particularly closely related species in terms of transformation compatibility and DNA base composition Thus the interrelations of the organisms described by *Petit* (1899) by *Scarlett* (1916) and by *Jones & Little* (1923) are clearly indicated If the strains of *Moraxella lacunata* employed (*Bovre* 1965 c) are representative of the organisms described by *Morax* (1896) and *Axenfeld* (1897), the transformation results (*Bovre* 1965 c) and DNA base data indicate that these organisms and those of Petit are varieties or types of the same entity This is in accordance with the opinion expressed by *Axenfeld* (1929) Another variety of the same entity seems to be the organisms isolated by *Ryan* (1964) from guinea pig conjunctiva (*Bovre* 1965 c)

An important implication is that the concept *Moraxella nonliquefaciens* (in its common sense) is heterogeneous in corroboration with the results of conventional tests performed by *Mannheim & Stenzel* (1962) Whether the original description of *Bacillus duplex non liquefaciens* (*Scarlett* 1916) was based on the fastidious ones with the closest relation to other moraxellae (as assumed above) or on strains similar to the 19116 51 group (growing fairly well on Hugh & Leifson s medium and feebly in citrate media see *Bovre* 1965 d) cannot be decided with certainty In either case the 19116 51 group is clearly distinct from the fastidious *Moraxella nonliquefaciens* The results of DNA base determinations show a very uniform deviation between the two and their transformation compatibility is very low The '19116 51 group should therefore be considered as a separate species probably with some taxonomic relation to moraxellae The name *Mima polymorpha* var *oxidans* has in some instances been used on strains probably belonging to the 19116 51 group but the same name has also been applied to dissimilar organisms (*Bovre* 1965 d) The name is not recommended for the 19116 51 group (see below)

It is known from preliminary investigations that there are oxidase positive immotile rods which have little or no compatibility with any of the oxidase positive rods mentioned including the "19116/61 group Thus the urease positive strain 7592/62 (*Bovre* 1964 b) and organisms received from the late Miss E O King may constitute another separate entity

Since the descriptions of *Bacillus duplex non liquefaciens* (*Scarlett* 1916) and *Mima polymorpha* var *oxidans* (*De Bord* 1942) both are inadequate and authentic strains are unavailable any attempt to prove their identity will inevitably be fruitless It is felt that *Mima polymorpha* var *oxidans* and the fastidious *Moraxella nonliquefaciens* are not identical since genetic studies as yet have provided no evidence of identity of the latter and strains with the former designation and also because *Mima polymorpha* var *oxidans* originally was described as luxuriantly growing with white colonies For discussion of the standing in nomenclature of the names *Mimeae Mima* and species epithets *Henriksen* (1963 b) and *Pickett & Manclark* (1965) should be consulted

Affinities within Neisseria The conclusion of *Catlin & Cunningham* (1964 a) that the nitrate negative strain *Neisseria catarrhalis* 4103 and *Neisseria cinerea* might be identical has not been verified (*Bovre* 1965 b) Although transformation between this strain and other *Neisseria catarrhalis* strains has given similar results in both investigations (in itself a good control of the strains employed and the reproducibility of the method) direct testing of *Neisseria cinerea* strains revealed their distinctiveness from strain 4103 and their probably close relations to *Neisseria flavescens* DNA base composition data are corroborative The strain 4103 may represent a species close to *Neisseria catarrhalis* Any consistent characteristic of this eventual species cannot be suggested with only one strain

There seems to be a rather close relationship between *Neisseria ovis* and *Neisseria caviae* Their DNA base compositions are identical These organisms also have some relations to *Neisseria catarrhalis* in terms of transformation and in biochemical and cultural tests (*Berger* 1962 *Bovre* 1965 b) Some taxonomic distance between the latter and the two former is indicated by their low transformation compatibility and by their deviating DNA base ratios However the suggestion that *Neisseria caviae* may be a close relative of the pigmented saccharolytic neisseriae (*LaMacchia & Pelczar* 1966) does not at all fit with the present investigation It [illegible] possible that strains called *Neisseria caviae* represent different [illegible] In that case it is important to note that the present study [illegible] the type strain of the species (*Bovre* 1965 b)

The DNA b[illegible] [illegible] similarities and absence [illegible] compatibilities between (*Neisseria catarrhalis* *Neisseria* [illegible] *ae* and (*Neisseria cinerea* *Neisseria flavescens*) [illegible] [illegible]tionship be

tween the two groups (see *Bøvre* 1965 b) It may therefore seriously be doubted whether all these organisms should be placed in one genus, as is now the case (*Breed, Murray & Smith* 1957) *Catlin & Cunningham* (1961) arrived at a similar conclusion as regards *Neisseria catarrhalis* and its distinction from group I neisseriae (see previous discussion of this report) Thus, results are largely corroborative and supplement each other on this point

Affinities between Moraxella *and* Neisseria The closeness in terms of overall DNA base composition and partly rather pronounced transformation compatibility of moraxellae and not yellow-pigmented, asaccharolytic neisseriae (including *Neisseria caviae*), indicate that the two assumed entities have important relations They should probably be placed in the same family of the present taxonomic system, as suggested by *Henriksen* (1952) on the basis of conventional criteria

Affinities between oxidase positive and oxidase negative organisms *Henriksen* (1952) proposed that the genus *Moraxella (Lwoff* 1939) should not be supplemented with oxidase negative organisms *Brisou & Morichau-Beauchant* (1952) and *Brisou, Morichau-Beauchant & Gimenez* (1953) shared the opinion that *Moraxella lwoffi* (*Audureau* 1940) does not belong to genus *Moraxella* *Piéchaud, Piechaud & Second* (1956), however, held the attitude that *Moraxella* includes oxidase negative species So far, the view of Henriksen and Brisou and collaborators seems correct

Close relationships across the boundaries between oxidase positive and oxidase negative organisms seem possible in terms of overall DNA base composition It is noted that the two *Achromobacter lwoffi* strains with greatest similarity to the "19116 51" group have the same G + C content as the latter (see *Bøvre* 1967 b) The results of transformation experiments, however do not support this eventuality There is no indication of a species (*Mima polymorpha*) with an oxidase positive and an oxidase negative variety, as first introduced by *De Bord* (1942) and as mentioned no indication that *Moraxella lwoffi* belongs to the same genus as the fastidious moraxellae It is not known, however whether genetic reactivity of the order observed between *Achromobacter* and the "19116 51" group, and between the former and *Neisseria catarrhalis*, is indicating some distant relationship Further studies on this point ought to have high priority

Comment A complete taxonomic proposal at this stage is considered premature Many of the above considerations are preferably left as working hypotheses for future investigations in the field

It may be argued that the proposed species entity is too rigidly delimitated and that DNA base similarity and transformation interactions of the order observed between the three unequivocal *Moraxella*

entities suggest species identity (still with the "19116/51" group as a separate species). This attitude would at least partly be in accordance with the opinion of numerous authors. The same theoretical line would lead to the suggestion that *Neisseria flavescens* and *Neisseria cinerea* represent the same species, which does not seem unreasonable. The exclusion of strain 4103 from *Neisseria catarrhalis* would be prevented. *Neisseria caviae* and *Neisseria ovis* also would possibly be considered as varieties of the same species. In other parts of the classification system, however, analogous deductions would lead to more radical changes, although still not very unreasonable. For instance, *Neisseria meningitidis* would be a variety of a species containing all hitherto examined yellow pigmented and/or saccharolytic neisseriae. Species distinction between pneumococci and some streptococci would be difficult to maintain. In this eventual alternative system the relations between moraxellae and the neisseriae in question would be even more suggestive.

However, a conservative attitude is considered the best alternative. One must take advantage of additional information which undoubtedly very soon may be made available.

Differences in conventional terms found between groups suggested on the basis of genetic compatibility and DNA base similarity should be followed up and supplemented for the ultimate construction of a useful key reaction system.

SUMMARY

A review has been given on genetic transformation and overall DNA base composition in taxonomy. Own transformation studies in *Moraxella*, *Neisseria* and some other organisms were summarized and discussed. DNA composition data of the material showed good correlation with the transformation results.

The main conclusions were:

1. *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying nonhaemolytic moraxellae (inclusive *Moraxella lacunata*) may be considered as three particularly closely related species.

2. A distinct group of *Moraxella nonliquefaciens*-like organisms, provisionally called the "19116/51" group, should be considered as a separate species, probably with some taxonomic relations to the unequivocal moraxellae. This species is in conventional tests indistinguishable from the fastidious *Moraxella nonliquefaciens*, except for its capacity to grow fairly well on Hugh & Leifson's medium and feebly in citrate media. Its G + C content in CsCl buoyant density determinations is of the order 43–43.5 per cent (40–42 per cent for *Moraxella nonliquefac*[illegible] with most strains at 41 per cent). Streptomycin resistance transformation between the "19116/51" group and unequivocal morax[illegible] has never been observed [illegible] of intrastrain transformation frequency, whereas [illegible] *nonliquefaciens*

always gives reciprocal ratios above 10^{-3} with the other two unequivocal moraxellae

3 Absence of genetic compatibility and DNA base similarity between the groups (*Neisseria catarrhalis*, *Neisseria ovis*, *Neisseria caviae*) and (*Neisseria cinerea*, *Neisseria flavescens*) may indicate uncorrect ordering of these organisms in one and the same genus *Neisseria cinerea* and *Neisseria flavescens* should be considered closely related

4 Overlap in terms of DNA base composition and partly also rather pronounced genetic transfer between moraxellae and asaccharolytic not yellow pigmented neisseriae suggest that the two groups have important taxonomic relations and that family distinction between them probably is too rigorous

5 There is at present no evidence of relationship between unequivocal moraxellae and oxidase negative bacteria, inclusive *Achromobacter* (*Moraxella*) *lwoffi* Weak interactions observed in transformation between *Achromobacter*, the "19116/51" group and *Neisseria catarrhalis* should lead to further investigations, however

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SEROLOGICAL PROPERTIES OF ANTISERA TO *NEISSERIA GONORRHOEAE* ANTIGENS

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Received 8.vii.66

In 1950 *Thomas & Mennie* (26) described the indirect haemolysis test, called by them the polysaccharide lysis test, for detection of antibodies in human sera against antigen extracted from gonococci. They proposed to substitute the conventional complement fixation test by the indirect haemolysis test in the diagnosis of gonorrhoea.

Chanarin (3) extracted 2 antigens from smooth strains of gonococci by alkaline hydrolysis of the bacteria. One antigen was detected by its ability to sensitize erythrocytes to the action of gonococcus antiserum. The antigen failed to react in a precipitin reaction and did not fix complement in the presence of antiserum. The second antigen was detected by its ability to fix complement in the presence of antiserum to gonococci. *Chanarin* suggested that the 2 antigens formed a complex antigen on the surface of the gonococcus.

In a previous work the author reported on the gonococcus antigen which sensitizes erythrocytes (19). The antigen was extracted following the procedure of *Chanarin* (3). The indirect haemolysis test was employed for the titration of antibodies against sensitized erythrocytes. The titre of the haemolysis test depended on the antigen dose employed for sensitization, the complement concentration used, and time of incubation. Antibodies against sensitized erythrocytes were found in 55 per cent of blood donor sera. The indirect haemolysis test afforded no advantage over the complement fixation test in the diagnosis of gonorrhoea. A rabbit antiserum and human sera also agglutinated sensitized erythrocytes. The titre of the antiserum in the indirect haemolysis test far exceeded that of the haemagglutination test.

Some properties of antisera obtained by immunization of rabbits with whole gonococci and with sensitized erythrocytes, have been studied. This paper presents the results.

MATERIAL AND METHODS

N. gonorrhoeae str[illegible]. Strain 8351/64 isolated from a [illegible] patient suffering from acute gonorrhoeal u[illegible]thritis was selected. The strain wa[illegible]erved and stored by

lyophilization Cultivation harvesting and preparation of the bacterial extract were performed as described previously (19)

Antisera Antisera against strain 8551/64 (designated anti Gc) were prepared according to the immunization schedule described previously (19) Antisera against the antigen which sensitizes erythrocytes (designated anti SNSE (sensitized normal sheep erythrocytes)), were obtained by immunizing 2 rabbits with sensitized ery throcytes The erythrocytes were sensitized as previously described (19), thoroughly washed and prepared as a 10 per cent suspension One ml was injected intravenously thrice weekly for four weeks As the antibody response was relatively weak a new series of three injections was given after an interval of 4 weeks The animals were bled 7 days after the last injection The sera were stored at —20° C Before use they were inactivated and absorbed with unsensitized sheep erythrocytes

Indirect haemagglutination and haemolysis tests The procedures described pre viously were followed except that the haemolysis test was modified when sera previously treated with mercaptoethanol or absorbed with gonococci were titrated Before complement was added the sheep erythrocytes in each tube were washed once and resuspended in 0 4 ml volumes

Complement fixation test When whole gonococci were employed as antigen the test was performed as previously described (19) Using extract of gonococci the procedure was similar except that fixation of the complement took place overnight at 4° C before incubation at 37° C

Precipitation tests Precipitation in capillary tubes (ring test precipitation) was performed according to *Haukenes* (11) Dilutions of the bacterial extract were layered over undiluted serum The results were recorded instantaneously and after ½ 1 2 and 3 hours incubation at room temperature

The technique used in the agar gel diffusion tests has been described elsewhere (12) The antigen wells were filled either with a suspension of gonococci con taining about 100 mg of bacteria (wet weight) per ml or with undiluted extract The serum wells were filled with undiluted serum The plates were kept at 37° C for 1 day followed by incubation at 4° C for 1 or 2 days The precipitation patterns were read daily

Agglutination of gonococci Agglutination of live gonococci and gonococci treated with heat or formalin was performed using a slide technique An 18 20 hours culture was harvested in saline One portion of the gonococci was resuspended in saline to a density of approximately 10^9 organisms per ml The second portion was boiled in a water bath for 60 minutes washed once and resuspended in a similar manner This washing was necessary to obtain maximal titre probably because antigens liberated to the fluid phase inhibited agglutination A third portion of gonococci was treated with 3 per cent formalin at room temperature for 60 minutes thoroughly washed and resuspended in saline Twofold dilutions of the antisera were prepared in saline from 1 2 One drop of each serum dilution was mixed with 1 drop of the bacterial suspension on a slide The slide was rocked for 10 minutes and left on the disk for 5 minutes The agglutination appeared as numerous fine granules The titre is given as the reciprocal of the highest serum dilution with marked agglutination

Absorption Samples of undiluted antisera were absorbed either with live gono cocci or with organisms treated by heat at 100° C for 60 minutes For the absorption of 0 1 ml of serum about 200 mg of bacteria (wet weight) were employed and the mixture was incubated at 4° C for 4 hours

The extract of gonococci was absorbed with unsensitized erythrocytes (22) Several absorptions with large doses of erythrocytes were needed Absorption was considered complete when the extract no longer sensitized erythrocytes to the action of an antiserum

Mixed agglutination The procedure was adapted from that described by *Tönder et al* (27) working with mixed agglutination with tissue sections One drop of a suspension of gonococci was placed in the centre of a coverglass The preparation was air dried fixed in formalin and washed according to *Hess et al* (13) The bacteria were sensitized by placing 1 drop of anti Gc diluted 1 4 on the preparation which thereafter was kept at room temperature for 60 minutes washed and air dried Microculture slides with a single concavity were filled with a 1 per cent sus pension of erythrocytes sensitized as usual with the bacterial extract The cover glass was placed in such a way that the bacterial preparation was submerged in the erythrocyte suspension The slides were inverted and kept at 4° C overnight They

were again turned over and the coverglass was tapped gently around the bacterial preparation to accelerate the detachment of passively adsorbed erythrocytes The preparation was then examined microscopically at a 100 fold magnification

Treatment with mercaptoethanol Equal volumes of undiluted antiserum and 0 2 M 2 Mercaptoethanol pH 7 4 were mixed kept at room temperature overnight and tested for activity (10) Proper controls were included

Gel filtration A 3 ml sample of anti Gc was absorbed with unsensitized sheep erythrocytes and applied to a Sephadex G 200 gel bed of 3 5 × 45 cm The gel filtration was performed according to *Flodin & Killander* (9) employing 0 05 M potassium phosphate buffer pH 7 3 containing 0 4 M NaCl and 0 02 per cent sodium azide The UV absorption of the effluent was recorded at 254 mμ in a Uvicord absorptiometer (LKB Produkter Stockholm Sweden) The fractions were tested for activity in the indirect haemagglutination and haemolysis tests

EXPERIMENTS AND RESULTS

Bacterial Agglutination, Complement Fixation, Indirect Haemagglutination and Haemolysis

In preliminary experiments the titres of the sera in the various tests were determined using varying concentrations of the antigens In the the final tests antigen concentrations were chosen so as to give maximal titres

TABLE 1

Titres of Anti Gc and Anti SNSE in the Various Tests

Serologic tests	Antigen	Anti Gc	Anti SNSE
Bacterial agglutination	Live gonococci	256	<2
	Boiled gonococci	128	32
Complement fixation	Whole gonococci	256	32
	Extract of gonococci	128	16
Indirect haemagglutination	"	512	64
Indirect haemolysis		8192	512

Table 1 shows the results obtained with anti Gc and anti SNSE Anti Gc reacted in the bacterial agglutination test with both live and boiled gonococci With boiled gonococci the titre seemed to be slightly lower in accordance with the finding of *Danielsson* (4 6) Anti SNSE did not react in the agglutination test with live gonococci but boiled gonococci were agglutinated The agglutination test with formalinized bacteria allowed no conclusion because the bacteria agglutinated spontaneously to a considerable degree No spontaneous agglutination occurred with live or boiled gonococci

Anti SNSE absorbed with unsensitized sheep erythrocytes was moderately anticomplementary presumably caused by soluble complexes of antigens and antibodies in the absorbed serum This was circumvented by absorbing the serum with sheep erythrocyte stromata and centrifuging at high speed Anti Gc and anti SNSE reacted in the complement fixation test no matter whether whole gonococci or the extract was the antigen (Table 1)

Twofold dilutions of unabsorbed extract and of extract absorbed with

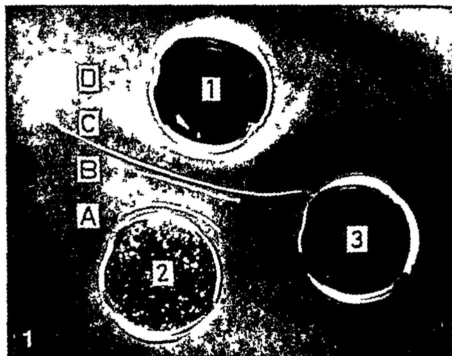


Fig 1

Precipitation pattern formed by gonococci and antisera Gonococci anti Gc and anti SNSE in wells 1 2 and 3 respectively Precipation lines A B C and D See text Line A and B artificially intensified

sheep erythrocytes were prepared The complement fixing ability of the samples in the presence of antiserum was determined using a constant amount of anti Gc The complement fixing activities of the samples were essentially similar thus showing that the extract contained one or more antigens in addition to the antigen which sensitizes erythrocytes

Both antisera reacted in the indirect haemagglutination and haemolysis tests and with considerably higher titres in the latter

Following absorption of anti SNSE either with live or boiled gonococci the antiserum no longer agglutinated heat treated gonococci and the reactivity with sensitized erythrocytes was abolished

The sera obtained from both rabbits immunized with sensitized erythrocytes behaved similarly in the various tests Preimmune sera were negative

Precipitation

Precipitation in capillary tubes with undiluted anti Gc was obtained with dilutions of both unabsorbed extract and extract absorbed with erythrocytes up to 1 128 When tested against undiluted anti SNSE precipitation appeared up to dilution 1 4 of the extract only

Suspension of gonococci and the extract were examined by double

diffusion in agar against anti Gc and anti SNSE Fig 1 shows the principal precipitation lines appearing with gonococci in the antigen well Undiluted anti Gc formed 4 precipitation lines referred to as A B C and D Line A formed near the serum well and tended to disappear into the serum basin Midway between the antiserum and antigen wells 2 relatively straight precipitation lines appeared (line B and C) The fourth precipitation line line D formed near the antigen well This line fused with the only precipitation line formed by anti SNSE

With extract in the antigen well anti Gc formed line A only No precipitation line was formed with anti SNSE and the extract

Mixed Agglutination

When smears of gonococci are incubated with antiserum antibodies react with antigens on the surface of the bacterial cell After removal of the serum fixed antibodies are detected with an indicator system which in this case was erythrocytes sensitized with the extract Antibodies bound to the bacteria also react with sensitized erythrocytes and thus function as a linkage between the erythrocytes and the bacterial smear The indicator system employed detects only the antigen which sensitizes erythrocytes

Fig 2 shows the picture of the microscopical readings at 140 fold and 560 fold magnifications The bacterial preparation is covered with erythrocytes while only few erythrocytes attach to the coverglass (Fig 2a) In accordance with the grading used by *Tonder et al* (27) Fig 2a shows a moderately positive reaction (+2) Fig 2b visualizes the arrangement of single erythrocytes at the periphery of the bacterial smear Erythrocytes adhere closely to aggregates of bacteria

In control preparations with sensitized erythrocytes and pre immune serum or unsensitized erythrocytes and antiserum no erythrocytes attached to the bacterial smears

Reduction by Mercaptoethanol Gel Filtration

The haemagglutinating activity of anti Gc and anti SNSE was only slightly influenced by treatment with mercaptoethanol

On the other hand the haemolysis titres were decreased to about the same as the haemagglutination titres The results indicated that antibodies against sensitized erythrocytes belong to both γM and γG globulins (8 10)

Fig 3 gives the results obtained by gel filtration of anti Gc The first peak of the protein curve corresponds to fractions containing mainly γM globulins the second one mainly γG globulins [illegible] This was verified by treatment of the fractions with mercaptoethanol

The antibody activity of the fractions was tested in the haemagglutination and haemolysis tests With most γG globulin fractions the titres of the hae

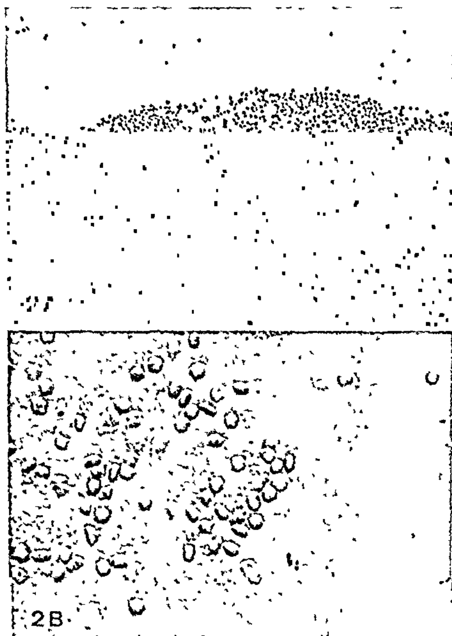


Fig 2

Mixed agglutination with a smear of gonococci on a coverglass

a 140 fold magnification The smear of gonococci is covered with erythrocytes, while only a few erythrocytes attach to the coverglass

b Appearance at 560 fold magnification at the periphery of the preparation Sensitized erythrocytes crowded around aggregates of bacteria

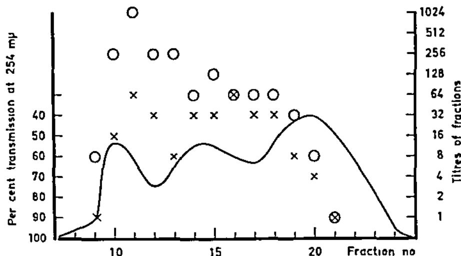


Fig 3

Serological activity of fractions obtained by gel filtration of rabbit anti Gc serum on Sephadex G 200 column
—— Per cent transmission at 254 mμ
× Titres in indirect haemagglutination test
○ Titres in indirect haemolysis test

molysis test was double the haemagglutination titre Most γM globulin fractions reacted with 16 times higher titres in the haemolysis test *i e* with titre difference of the same order as with unfractionated anti serum

TABLE 2

Titres in Haemagglutination and Haemolysis Tests of Mixtures Containing Varying Quantities of a γM and a γG Globulin Fraction of Antiserum

Mixtures titrated Volumes of γM fraction	γG fraction	Indirect Haemagglutination	Indirect Haemolysis
0	10	32	64
2	8	32	128
4	6	64	256
6	4	64	256
8	2	64	512
10	0	64	1024

The influence of antibodies belonging to γM globulin and γG globulin on the indirect haemagglutination and haemolysis tests was investigated by varying the concentration of the globulins Samples from 1 γM and 1 γG globulin fraction were mixed as shown in Table 2 and the reactivity of the mixtures against sensitized erythrocytes was determined The haemagglutinating capacity of the 2 fractions was only

slightly different, and hence the titres of the mixtures in the haemagglutination test increased from 32 to 64 only with increasing volumes of the γM-fraction in the mixtures On the other hand, the titres in the haemolysis test increased from 64 to 1024 as the volumes of the γM-fraction increased The results indicate a different behaviour of γM- and γG-globulin antibodies in the indirect haemolysis test

DISCUSSION

Rabbits immunized with sensitized erythrocytes formed antibodies against the adsorbed antigen However, the antibody response was relatively weak as compared to that obtained when live gonococci were injected

Anti-Gc reacted in the bacterial agglutination test with live and heat treated gonococci (boiled for 60 minutes) Anti-SNSE did not agglutinate live gonococci, but boiled gonococci were readily agglutinated The antigen reacting with anti SNSE is thermostable Anti-SNSE thus resembles antisera against somatic O antigens of certain enteric bacilli, which do not agglutinate unless the organisms are treated with heat (16) *Boor & Miller* (1) immunized rabbits with glycolipid from gonococci and meningococci and obtained antisera which exhibited excellent precipitating but mediocre agglutinating power against homologous strains *Deacon* (7) observed inagglutinability of freshly isolated strains of gonococci in antisera prepared against heat treated cultures He suggested that K-like antigens (labile surface antigens) act as a protective cover over underlying, thermostable, somatic antigens, and prevent agglutination The K antigen was readily lost in subcultures

The gonococcus strain employed in this study had been subjected to several subcultures and hence a K-antigen was probably no hinderance for agglutination with anti-SNSE The reactivity of anti-SNSE against sensitized erythrocytes disappeared after absorption of the antiserum with live gonococci The antibodies in anti-SNSE thus reacted with live gonococci but treatment of the bacteria with heat was necessary to render them agglutinable by anti-SNSE

Chanarin (3) did not demonstrate complement-fixing activity of the erythrocyte sensitizing antigen in the presence of antiserum However, complement was fixed in the presence of anti SNSE regardless of whether the extract or whole gonococci was the antigen Unchanged complement-fixing activity in the presence of anti Gc of extract absorbed with erythrocytes, established the existence of at least one additional antigen in the extract This finding agrees with the observations of *Chanarin* (3)

Precipitation in capillary tubes between anti Gc and unabsorbed and absorbed extract, furnished further evidence for the existence of additional antigen(s) in the extract This antigen(s) failed to adsorb to erythrocytes In gel diffusion tests, the additional antigen formed a

precipitation line line A near the serum well with anti Gc *Chanarin* (3) did not demonstrate precipitation between the extract of gonococci and various antisera

Only weak precipitation occurred between anti SSSE and the extract in capillary tubes and no precipitation line was formed in gel diffusion tests Thus the sensitizing antigen in the extract seemed to be a weak precipitinogen only The precipitating property of anti SSSE was clearly demonstrated in gel diffusion tests with whole gonococci A precipitation line line D formed near the antigen well *Danielsson* (6) divided the precipitation lines formed by gonococci treated with ultrasonic and antisera against formalin killed gonococci into 4 groups referred to as A B C and D The precipitation line formed by anti SSSE and whole gonococci probably corresponds to group D

Two findings indicate a superficial location in the bacterial cell of the erythrocyte sensitizing antigen 1 The results obtained in the mixed agglutination test The mixed agglutination procedure can detect only antigens localized on the cell surface not those present inside the cell (27) 2 Disappearance of reactivity against sensitized erythrocytes of antisera absorbed with live gonococci

Mixed agglutination technique has mainly been employed in studies on tissue antigens and has previously not been reported with bacterial preparations on slides The principle of mixed agglutination resembles that of immunofluorescent staining which has come into use as a method for demonstrating gonococci in smears from patients (5 7, 23) A similar application of the mixed agglutination technique probably remains a topic for investigation

In the indirect haemagglutination and haemolysis tests anti Gc reacted with considerably higher titres than anti SSSE (Table 1) The titres of the sera in the haemolysis test exceeded the haemagglutination titres 16 and 8 times respectively Essentially similar differences between haemagglutination and haemolysis titres were found with sheep erythrocytes which had adsorbed antigen from *Escherichia coli* and *Salmonella* and homologous rabbit antisera (20 21)

These findings indicated that antibodies against sensitized erythrocytes belong to both γM and γG globulins 1 Reduction of antiserum by mercaptoethanol affected the reactivity of the serum to a considerable degree (8 10) 2 After gel filtration of antiserum activity appeared in fractions known to contain γM and γG globulins respectively (9 17) Antibodies against bacterial antigens of γM class have been found in antisera from several animal species including rabbits (14 15 18)

With most γM fractions the titre of the haemolysis test was 16 fold the haemagglutination titre as compared to a twofold difference with most γG fractions In titration of γM class antibodies the sensitivity of the haemolysis test is thus quite superior to that of the haemagglutination test while the sensitivity of the haemolysis test is only moderately superior in titration of γG class antibodies The results agree with the

findings of greater haemolytic capacity of γM- than of γG class antibodies in rabbit antisera against sheep erythrocytes (24, 25, 28)

Weinrach et al (28) attributed the difference in haemolytic capacity to a different mechanism of complement fixation by antibodies belonging to γM- and γG globulins Recently *Borsos & Rapp* (2) concluded that a single molecule of γM globulin at the cell surface is sufficient to bind 1 molecule of C'1a while at least 2 γG globulin molecules are needed

The difference between the titre of the haemagglutination and haemolysis tests depends on the relative amounts of γM- to γG class antibodies Accordingly, one would expect the titre difference to vary with different antisera and with sera obtained at various intervals through the immunization period

Investigations currently in progress, indicate that anti SNSE contains antibodies of at least 2 different specificities

SUMMARY

Serologic properties of antisera to whole gonococci (anti Gc) and anti sera obtained by injections of rabbits with erythrocytes which had adsorbed a *Neisseria gonorrhoeae* antigen (anti-SNSE), were investigated

Anti Gc agglutinated live and boiled gonococci Ant-SNSE agglutinated boiled gonococci only Anti SNSE and its homologous antigen exhibited complement fixing activity In gel diffusion tests, anti Gc and whole gonococci formed at least 4 precipitation lines, while anti-SNSE formed a single precipitation line near the antigen well

Loss of reactivity against sensitized erythrocytes of antiserum absorbed with live gonococci and the results obtained in mixed agglutination tests, indicated a superficial location in the bacterial cell of the erythrocyte sensitizing antigen

Antibodies against sensitized erythrocytes belong to γM- and γG globulins In the indirect haemolysis test the antisera reacted with far higher titres han in indirect haemagglutination This titre difference was attributed to superior sensitivity of the indirect haemolysis test in titration of γM-class antibodies

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Acta path et microbiol scandinav 69 156 157, 1967

BRIEF REPORTS

STUDIES ON THE TOXIC EFFECT OF TOXOTOXIN AND *TOXOPLASMA* LYSATES

By *Filip K Pettersen*

In 1950 *Weinman & Klatchko* reported that a cell free peritoneal exudate from mice previously infected with *Toxoplasma gondii* RH strain was toxic to mice when injected intravenously This toxic effect which was ascribed to toxotoxin was enhanced by incubating the exudate at 56° C for 30 minutes (*Woodworth & Weinman* 1960) and destroyed by the action of trypsin

In 1965 *Fulton* working with cotton rats reported that the effect of toxotoxin was also destroyed by the action of hyaluronidase suggesting that the lethal effect was due to a protein hyaluronic acid complex

The obvious contamination of *Toxoplasma* antigen preparations with mouse proteins prompted the following investigation the aim of which was to reveal possible toxicity of the contaminants produced by the mouse

The exudate from 20 g white mice infected with *Toxoplasma gondii* RH strain 5 days previously was used The parasites were removed by centrifugation at 500 G for 45 minutes The cloudy supernatant was recentrifuged at 37000 G for 10 minutes giving a clear supernatant liquid which contained precipitating antigens of both *Toxoplasma* and mouse origins The liquid was incubated at 56° C for 30 minutes and left at 4° C 48 hours later a considerable clot had formed which was obtained as the sediment by centrifugation at 37000 G for 10 minutes This was dissolved in demineralized water and filtered on a Jenaer G5 sintered glass filter (*Ogston* 1950) The residue was redissolved in demineralized water twice and filtered giving finally a white residue which was dissolved in 0 9 per cent sodium chloride solution and centrifuged at 37000 G for 10 minutes The supernatant had a lethal effect on mice which corresponded to that of the original cell free exudate Unless otherwise stated all the steps in the above procedure were performed at 4° C

The toxic solution thus obtained was tested in the agar gel diffusion technique (*Wadsworth* 1962) in which it formed several precipitin lines against rabbit anti mouse serum whereas no precipitin lines were detected against a human anti *Toxoplasma* immunoglobulin preparation

Incubation with rabbit anti mouse serum at 37° C removed the toxic material from this solution whilst incubation with the human anti *Toxoplasma* immunoglobulin preparation merely diluted the toxic solution

Thus the toxic material appeared to be of mouse origin rather than a product of the *Toxoplasma* parasite

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In 1964 *Lunde & Jacobs* described the toxic effect of *Toxoplasma* lysates on rabbits The parasites were obtained from mouse peritoneal exudate and the toxic effect ascribed to a protein

In this laboratory the parasites obtained as above were suspended in 0 9 per cent sodium chloride solution and filtered without suction through a Jenaer G1 sintered glass filter Centrifugation of the filtrate at 500 G for 20 minutes gave a sediment containing the parasites The sediment was suspended in demineralized water and left at 4° C until the next day when the suspension was centrifuged at 37000 G for 10 minutes giving a clear red [illegible] lysate Rabbits injected intravenously with this lysate died within 18 h [illegible] th minimum lethal dose was

Received 24 vi 66 from *the Department of Tox* [illegible] *Viral Diseases (Head J Chr Siim M.D.) State Serum Institute C* [illegible] *Denmark*

In part aided by grants to the Department [illegible] sis and Viral Diseases from *King Christian X Foundation* and the [illegible] *Service Research Grant Foundation* (AI 1741) Bethesda Md

found by the Folin phenol reagent (*Lowry* 1951) to be 60–75 µg protein per kg body weight, which agreed with the findings of *Lunde & Jacobs*

The toxicity of these lysates diminished rapidly on standing at temperatures above the freezing point Storage at —18° C caused some precipitation in the lysates and a solution stored thus for a week had lost more than half of its toxic effect When however, the lysate solutions were made isotonic before storage at —18° C, no precipitate was formed and the toxic effect was stable over a period of 6 months

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kg
Toxoplasma origins

The eluate was incubated with 20 per cent by volume of a human anti Toxoplasma immunoglobulin preparation for 3 hours at 4° C and centrifuged The supernatant was toxic to rabbits as before However, when the same volume of the eluate was

and 5 rabbits previously immunized with whole mouse blood The results are given in the table

TABLE

Intravenous Injection of Toxoplasma Lysate in Rabbits

	i v dose ml/kg	time lapse in hours before death
Normal rabbits	0 3	< 18
	0 3	< 18
	0 3	< 18
	0 3	< 18
Immunized rabbits	0 6	> 74
	0 6	24
	0 4	> 26
	0 4	< 18
	0 3	survived

The immunization of the rabbits which was started 8 months previous to this experiment consisted of 6 × one ml intramuscular doses of mouse blood glucose citrate solution and Freund s complete adjuvant (one third of each by volume) injected with about 4 weeks intervals Apparently this immunization the degree of which was not tested afforded some protection against the toxic effect of the *Toxoplasma* lysate

In view of these results and those obtained by *Lunde & Jacobs* this toxic material might prove to be of mouse origin as well as a product of the *Toxoplasma* parasite

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Acta path et microbiol scandinav 69, 158 159, 1967

ARTERIOSCLEROSIS INDUCED BY HYPOXIA

By *I Lorenzen* and *P Helin*

In the theories on the aetiology and pathogenesis of arteriosclerosis the effects of anoxia and hypoxia on the arterial wall have been suggested as factors of importance (*Hueper* 1944) We have been able to demonstrate the development of severe arteriosclerosis in the rabbit aorta following exposure of the animals to repeated periods of hypoxia through three weeks Male albino rabbits, 5 months of age, weighing about 3 kg were placed in a plastic box, one at a time The box was covered with plastic foil and air was sucked off through a valve in the bottom Via another valve in the side of the box pure nitrogen was then inflated until the animal became unconscious and had convulsions This happened after an average of 50 seconds whereupon the inflation of nitrogen was stopped and atmospheric air inflated instead About 30 seconds later the rabbit regained consciousness and was able to move Now and then however artificial respiration had to be given Immediately after, nitrogen was inflated again thus repeating the procedure. in this way, each animal was exposed to two hypoxic periods in one séance The entire procedure was performed twice daily
The study involved 10 experimental animals and 10 controls The controls were subjected to exactly the same procedure as the experimental animals with the exception that atmospheric air was inflated instead of nitrogen Three weeks after start the rabbits were killed by intravenous injections of 300 mg of phenobarbital

Results

In 7 out of 10 experimental animals pronounced gross alterations were observed The changes consisted of round or elongated hard white plaques mainly localized to the thoracic aorta In two of the animals the entire thoracic part of the aorta was involved and converted into a stiff tortuous tube Also aneurysms were observed In two controls a few small round plaques were observed in the ascending part of the aorta immediately above the aortic valve These changes were similar to the spontaneous alterations observed in previous studies (*Lorenzen* 1963) The aortae of the other controls were perfectly normal Changes similar to those observed in the experimental animals used in the present investigation were not observed in any of the 300 controls used in earlier studies Microscopic studies of the aortae of the the experimental animals showed scattered necrotic calcified lesions in the inner third of the media These lesions were surrounded by accumulations of amorphous intercellular substance staining metachromatically with toluidine blue indicating acid mucopolysaccharide, The accumulations of metachromatic substance were also observed between the elastic membranes outside the areas of the necrotic lesions Furthermore collagen deposits were noted partly around the calcified lesions and partly more diffusely scattered in the ground substance (van Gieson Hansen connective tissue stain) A cellular reaction involving fibroblasts and lymphocytes was observed around the necroses The elastic membranes of the media showed stretching splitting and fragmentation In a few animals a slight cellular thickening of the intima was seen usually in the area of medial changes Two of the control animals showed fibrotic calcified nodules in the subintimal part of the media corresponding to the gross changes mentioned above and to the spontaneous type of lesions described previously The arteriosclerotic changes induced by hypoxia are of precisely the same type as the changes previously induced in rabbits by injections of adrenaline and L-thyroxine (*Lorenzen* 1966) The changes are related to the

Received 17 xi 66 from the *University of Copenhagen Department of Dermatology with Connective Tissue Research Laboratories* (Professor G Asboe Hansen, M D), Rigshospital Copenhagen

'Monckeberg-type" of human arteriosclerosis They may be induced by hypoxia *per se*, or they may be be the results of factors which are secondary effects of hypoxia, for example a stimulated release of catecholamines

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Acta path et microbiol scandinav *69*, 159–160, 1967

A REEVALUATION OF THE CHRONIC BIOLOGICAL FALSE POSITIVE PHENOMENON WITH THE FLUORESCENT TREPONEMAL ANTIBODY ABSORPTION TEST

By *A. Lassus, K K Mustakallio K Aho* and *T Putkonen*

A *negative result of the treponema pall immobilization (TPI) test has been relied* upon as the most impor
the serological tests for
patients with clinically
reactive in the fluoresc
which the serum is first absorbed with Reiter treponemes to remove the group specific treponemal antibodies (1) This prompted us to retest sera from a series of patients classified as chronic BFP-reactors in the previous reports from our clinic (3 4, 5).

Sera from 57 such patients were available for study Nine of them had definite systemic lupus erythematosus (2) The FTA ABS test was performed according to the provisional technic published in 1965 by the Venereal Disease Research Laboratory of the Communicable Disease Center, Atlanta

Fifty of the 57 sera nonreactive to TPI were also negative in the FTA-ABS test, including the sera from the SLE patients One serum was reactive (2+) and six weakly reactive (1
is presented in the
the 50 cases nonre
whereas among the
41 The patients,
examined for 25 or
nective tissue dise
criteria, one of the 7 patients had definite rheumatoid arthritis

Received 1 xii 66 from the *Department of Dermatology University Central Hospital* Snellmaninkatu 14 Helsinki 17 Finland and from the *State Serum Institute* Helsinki Finland Supported by a grant from the *Sigrid Jusélius Foundation* Helsinki Finland

TABLE

Treponema Pallidum Immobilization Negative Cases with Reactivity to the Fluorescent Treponemal Antibody Absorption

Case	Sex	Age	Admission diagnosis	History	FTA ABS
1	♂	59	allergic eczema	drug eruption	reactive
2	♂	44	allergic eczema	—	weakly reactive
3	♀	76	positive STS	thrombophlebitis	weakly reactive
4	♂	65	seborrhoic eczema	pleurisy	weakly reactive
5	♀	62	positive STS	rheumatoid arthritis	weakly reactive
6	♀	54	infectious eczema	photosensitivity	weakly reactive
7	♂	58	lichen ruber	myalgia thrombophl	weakly reactive

It is impossible to prove that the TPI negative cases of the present series who showed a weakly reactive FTA ABS test did have latent syphilis. However, the available evidence of these patients as compared with the whole series does not contradict this possibility and we probably are safe in concluding that these patients should not be regarded as definite BFP reactors

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Acta path et microbiol scandinav 69 161–178 1967

The Connective Tissue Laboratory, Department of Dermatology, University of Copenhagen Rigshospital Denmark

EPIPHYSEAL GROWTH ZONES IN ACUTE LATHYRISM

Histochemical Study Electrophoresis of Cartilage and Bone

By

E BERNTSEN

Received 2 vii 66

The growth apparatus of tubular bones represent a well-defined anatomical and functional region, and, like other organ systems of the body, it is susceptible to the influence of a number of drugs interfering with the growth process

Since epiphyseal cartilage and spongious metaphyseal bone plates are integrating parts of the growth zone, both of these components were included in this study

Among compounds affecting the growth zones are lathyrogenic substances (*Selye* 1957) The present paper describes a lathyritic model, qualitative changes in growth zones and electrophoresis of cartilage and bone

MATERIAL AND METHODS

Throughout experimental studies on lathyrism albino rabbits in their third week of life weighing 200–250 grams were employed Animals and respective mothers were caged batchwise with free access to food and water Control and experimental animals were grouped by weight irrespective of sex at the start of the experiment, so that average body weights of the two groups were as close as possible

A 6 per cent sterile solution of aminoacetonitrile (AAN) Fluka AG Switzerland was made up and adjusted to pH 7 3 7 4 with NaOH A dosage of 15 mg per 100 g body weight per day through 4 days had been found suitable in preliminary experiments Injections were given subcutaneously into the back of the neck, while the controls received a corresponding volume of physiological saline

Carrier free radiosulphate in aqueous solution (The Radiochemical Centre Amersham), was diluted with 0 04 per cent Na_2SO_4 to a final conceentration of 1 mCi per ml

Animals were sa rifice [illegible] on the [illegible]ay after the last injection by an intraperitoneal overdose of Nembutal® [illegible]rom bones of the four extremities epiphyseal cartilages of proximal humeru[illegible] [illegible]tal radius and ulna, distal femur and proximal tibia were dissected out under a [illegible] microscope By circumcising the periosteum along the epiphyseal li[illegible] [illegible]physis was separated from metaphysis Cartilage was gently scraped off [illegible] m[illegible]taphyseal surface and epiphyseal "cap After splitting the bone shafts l[illegible]ng[illegible] [illegible]y marr[illegible]w was rem[illegible]ved a[illegible]d metaphysis surface cleared of possible cartila[illegible] [illegible]nants The radicalism [illegible]f this pr[illegible]cedure was controlled by microscopy [illegible] [illegible]us metaphyseal bone wa[illegible] then curetted [illegible]ut down [illegible] the level of the cortex O[illegible] [illegible]n[illegible]taphysis plates from [illegible]rresponding epiphyseal cartilages

Aided by grant fr[illegible] the *Danish Rheum[illegible] [illegible]s[illegible]ciation* and the *D[illegible]sh State Research Foundati[illegible]n*

were removed Samples were weighed immediately after dissection and dried to constant weight over P_2O_5 in an Edwards tissue drier at a pressure of less than 0 5 mm Hg After determination of dry weight, cartilage was crushed to a powder of fairly uniform particle size in a small glass grinder Bone was defatted for one hour in ethanol and for one hour in ether on a mechanical shaker, dried again to constant weight and powdered in a Wiley Mill (micromodel) using a 60 mesh sieve Decalcification was not performed

Samples for histological examination were taken from the distal part of the ulna, fixed for 24 hours in a mixture of 8 per cent cetylpyridinium chloride and 10 per cent neutral formalin (*Conklin* 1963), paraffin embedded, sectioned and stained with Toluidine blue 0 05 per cent at pH 5 for 3 min and with silver nitrate according to v Kossas method counterstaining with haematoxylin

The electrophoretic procedure described by *Marcl mann & Brunish* (1965) was followed Five mg of cartilage powder or 25 mg of bone powder suspended on a Vortex mixer with 200 μl or 100 μl of 0 5 N NaOH, respectively After 24 hours at 4° C samples were centrifuged and 1 μl of cartilage supernatant of 2 μl of bone supernatant were applied to cellulose acetate strips Besides Alcian blue stain for mucopolysaccharides, proteins were stained with 0 0025 per cent nigrosin (w/v) in 2 per cent acetic acid In some animals approximately 1 μCi of $^{35}S\text{-}SO_4$ per gram body weight had been injected intraperitoneally on the day of the third AAN or saline injection Autoradiograms were prepared by exposing strips on a Kodex® x ray film for 3 5 weeks

RESULTS

A typical lathyritic syndrome (*Selye* 1957) was produced The first symptom to appear was the ruffled fur, followed by drooping of the ears and a waddling gait with abducted upper extremities When at rest, animals sat on the rump rather than being supported by the lower extremities While controls gained weight progressively, the weight increase of experimental animals was arrested

At autopsy, the skin appeared thin, fragile and cut more easily During removal of bones, a marked muscular atrophy was noted together with diffuse intramuscular bleedings Proximal humeral and distal femoral epiphyses were invariably the most severely affected (*Ponseti & Shepard* 1954), occasional slipping always taking place at these sites

During dissection, control epiphyseal cartilage presented itself as a firm greyish material (Fig 1a b) resting upon a regular metaphyseal bone plate (Fig 3) Cartilage of experimental animals (Fig 2a, b) appeared loose and swollen, having an oedematous appearance with a definite haemorrhagic tinge It had a jelly-like consistence, in severe cases amounting to haemorrhagic liquefaction

The curved metaphyseal plateaus were moulded into more flattened surfaces, and metaphyseal height was reduced to about one third, presenting a disorderly bone structure (Fig 4) A severe osteoporosis (Fig 2b) was noted in the shaft near the growth zone lathyritic bones being definitely more fragile Periosteum was thickened, oedematous and easily detachable (Fig 2b) No fixed kyphoscoliosis was observed, neither did bowing of extremities nor exostoses develop The thoraco-abdominal content, in particular the aorta, appeared normal Costochondral joints were swollen



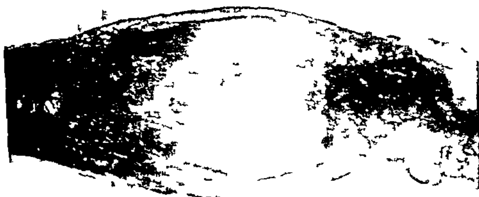


Fig 1

a Control rabbit D tal en l u d apl p nt ng lef C h z n c ere l b periosteum 1 — ag m a l o de ? — an n m cart lage pla e to se on la a n erm na la e of a situate l between 2 an l 3 6

b Same a F g 1a but pe h d l all h ugh a ap l in i n Arrow numl er a F


1
3
2
2 A


1
2
2 B



Fig 3

Control rabbit Median section through the upper end of the tibia Bone marrow and the entire epiphysis including the cartilage plate has been removed Uniform structure of the spongiosa (× 6)

Fig 2

a Lathyritic rabbit Distal end of lna diaphysis [illegible] ng left The entire growth zone is swollen Periosteum is ntact Arrow numb as in Fig 1 a (× 6

b Same as Fig 2 a but with [illegible] steum pushed istally thr ugh a [illegible] haped incision 1 — cartilage metaphysis border 2 transiti n fr m [illegible] germinal cell layer of the artilage plate 1 se of the [illegible] slipping periosteum coud n t be pushed [illegible] distally a [illegible] n tary ossification centre Some cartilagin us ma [illegible] is adher ng [illegible] steum Osteoporosis swelling of epiphyseal cart l [illegible] and oed na [illegible] teum is visualized (× 6)



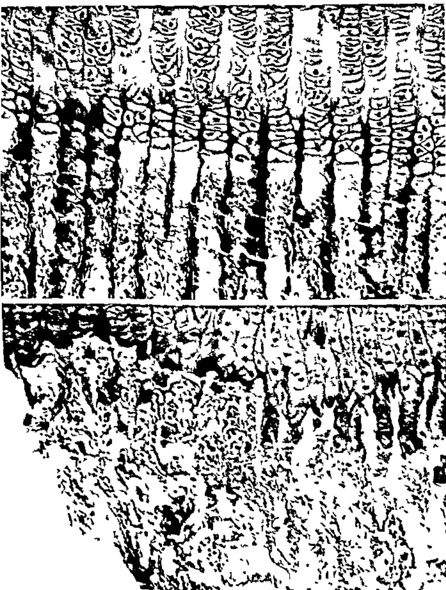
Fig 4

Lathyritic rabbit Median section through the upper end of the tibia Marrow and epiphysis removed as in Fig 3 Metaphysis height is greatly reduced and spongiosa structure is irregular (× 6)

In one animal regression was followed up to the fifth day and in another animal up to the ninth day after withdrawal of AAN After a few days body weights started to increase quite rapidly the fur regained its normal lustre but drooping ears and abducted upper extremities persisted in both animals

HISTOLOGY

Compared to control cartilage (Fig 5) the lathyritic cartilage plate (Fig 6) appeared swollen with periosteum bulging outwards An increased proliferation of cells of the maturing zone was observed Cell clusters were noted at the periphery where ground substance was also more abundant than in the central parts of the plate A proper hypertrophic zone could not be recognized Extracellular substance appeared



Figs 7 and 8

Cartilage metaphysis border showing regular formation of
dine blue stain (× 12)

Cartilage metaphysis border showing complete disorgani
formation Toluidine blue stain (× 125)



Fig 6

Lathyritic rabbit Zones as indicated in Fig. 5 Maturing zone is widened, there is no definite hypertrophic zone the cartilage metaphysis border is irregular and the metaphysis disorganized Toluidine blue stain (× 32)

When following the evolution of histological alterations from day to day, it was evident that after first AAN-dose, changes had occurred at the cartilage-metaphysis border The usual uniform pattern of formation of primary spicules was disturbed, leaving the junction with irregular and defectively formed trabeculae (Fig 11) Five days after withdrawal of AAN, this junction was still in a state of complete derangement (Fig 12), but after nine days without the compound an almost normal borderline was seen, with slender, regular and uniform trabeculae (Fig 13)

ELECTROPHORESIS

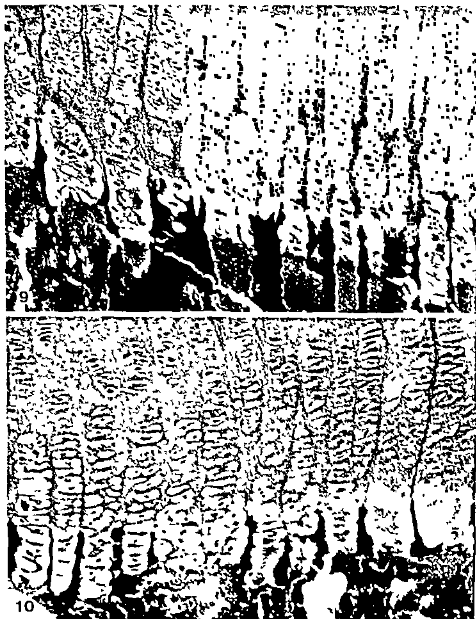
Both in control and experimental cartilages (Fig 14), a distinct chondroitin sulphate (ChS)-band and a distinct though much fainter hyaluronic acid (HA)-band were detected, fractions from controls and experimental animals appearing equally strong Besides two faint and inconstant bands were observed, one situated between ChS- and HA-



Figs 7 and 8

Fig. 7. Control rabbit. Cartilage-metaphysis border showing regular formation of [illegible]

[illegible] showing complete disorganization [illegible] stain (× 125).



Figs 9 and 10

Fig 9 Control rabbit Calcium is concentrating around hypertrophic cells towards the cartilage metaphysis border V Kossa stain (× 125)

Fig 10 Lathyritic rabbit Compared to Fig 9, a uniform calcification pattern cannot be recognized V Kossa stain (× 125)



Fig 11

Cartilage metaphysis border of a lathyritic rabbit 24 hours after a single dose of AAN Irregular formation of spicules Toluidine blue stain (× 125)

bands the other between HA and the starting line In metaphyseal bone, the same two major bands were identified (Fig 15) Control and experimental ChS bands again appeared equally strong whereas the HA band of lathyritic samples was definitely stronger than that of control samples

Both in cartilage and bone samples ChS-bands were of a slightly slower mobility than standards Some material was retained at the starting line, especially in case of the bone samples which were also the more viscous No protein spots were seen except at the starting line Autoradiograms showed radioactivity of cartilage as well as bone samples to be located in the ChS bands exclusively (Fig 16 17)

DISCUSSION

In establishing the lathyritic syndrome it was intended to keep the dose as low and the injection period as short as possible Since variations in food intake must be expected in lathyrism subcutaneous administration rather than ingestion was preferred (*Selye* 1957) By keeping the duration of the experiment short interference with repair processes was restricted (*Karn[illegible]sky* 1[illegible]61) and the risk of nutritional deficiency minimized (*Kuhlmann* 19[illegible])

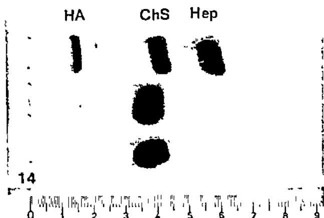
By the dissection technique described [illegible] was assured that nothing but epiphyseal cart[illegible] and metaph[illegible] spongiosa was [illegible] Therefore all layers [illegible] were represent[illegible]

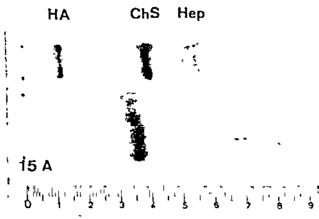


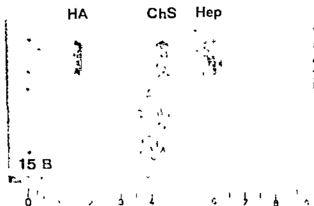
Figs 12 and 13

Fig 12 Cartilage metaphysis border of a lathyritic rabbit given AAN through 4 days and then sacrificed 5 days after withdrawal of the compound Formation of spicules is still completely disorganized Toluidine blue stain (× 125)

Fig 13 Cartilage metaphysis border of a lathyritic rabbit given AAN through 4 days and then sacrificed 9 days after withdrawal of the compound Formation of spicules is almost normal Toluidine blue stain (× 125)







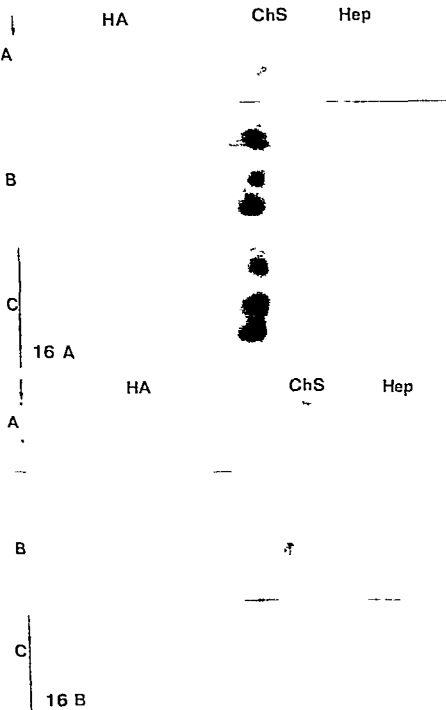
(For text see p. [illegible])



Figs 12 and 13

Fig 12 Cartilage metaphysis border of a lathyritic rabbit given AAN through 4 days and then sacrificed 5 days after withdrawal of the compound. Formation of spicules is still completely disorganized. Toluidine blue stain (× 125)

Fig 13 Cartilage metaphysis border of a lathyritic rabbit given AAN through 4 days and then sacrificed 9 days after withdrawal of the compound. Formation of spicules is almost normal. Toluidine blue stain (× 125)


HA
ChS
Hep
A
B
C
16 A
HA
ChS
Hep
A
B
C
16 B

HA ChS Hep

C

17 A

HA ChS Hep

A

B

C

17 B

That electrophoresis should show the presence of chondroitin sulphate was expected (*Dziewiatkowski* 1957), but as the standard was not chemically well defined, the type of chondroitin sulphate found could not be identified The presence of hyaluronic acid seems to be established This concurs with the chemical demonstration of hyaluronic acid in epiphyseal cartilage (*Hjertquist* 1964) The finding of the same two types of mucopolysaccharides in cartilage and bone lends some support to the view that metaphysis mucopolysaccharides may, at least in part be derived from epiphyseal cartilage (*Dziewiatkowski* 1957) The appearance of a stronger hyaluronic acid band in lathyritic metaphysis samples was reproducible, but should be regarded only as circumstantial evidence of an increase of this substance Although scanning of the strips (*Marckmann & Brunish* 1965) was also done, this procedure remained unreliable as quantitative assessment The significance of the faint intermediate bands is uncertain, since they were not constant

SUMMARY

Changes in epiphyseal cartilage and metaphyseal spongiosa of extremity growth zones of 2–3 week old rabbits rendered acutely lathyritic with aminoacetonitrile are described, macro- as well as microscopically Attention is particularly drawn to the disorganized endochondral ossification at the cartilage metaphysis border Electrophoresis on cellulose acetate strips of alkali extracted mucopolysaccharides from epiphyseal cartilage and metaphyseal spongiosa indicates the presence of chondroitin sulphate and hyaluronic acid in both types of tissue

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Fig 17

a Metaphyseal bone [illegible] ntrol sample
b Metaphyseal b[illegible] lathyritic sample

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Acta path et microbiol scandinav 69, 179–184 1967

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MALIGNANT MELANOMAS OF THE EYE AS STUDIED WITH A SPECIFIC FLUORESCENCE METHOD

By

B EHINGER, H OLIVECRONA and H RORSMAN

Received 8 viii 66

Dopa, 3,4-dihydroxyphenylalanine, is regarded as an important intermediary product in the synthesis of melanin (14, 11, 12) In model experiments dopa condenses with formaldehyde to form a strongly fluorescent product (4) Normal melanocytes and cells of pigmented naevi and malignant melanomas display fluorescence (8, 6) after a special formaldehyde treatment (3) It was assumed that the observed fluorescence was caused by dopa (6) Support for this assumption was obtained from the recent demonstration of dopa in malignant melanomas (5) In regard to the origin in common with the melanin producing cells in the skin and in the choroid of the eye an investigation of the fluorescence characteristics of ocular melanomas was of obvious interest

MATERIALS AND METHODS

The material comprises ocular melanomas from 11 patients 8 women and 3 men aged 37 to 75 years Immediately after enucleation about half of the tumours were quick frozen in propane cooled by liquid nitrogen freeze dried, and then treated with formaldehyde according to the method of *Falck & Hillarp* (3, 4 7) Controls for autofluorescence were obtained by excluding the formaldehyde in the treatment Sections were obtained at 6 8 μ and analysed in the fluorescence microscope (7) For ordinary light microscopy fixation was performed in formalin and the sections were stained in haematoxylin and eosin Classification according to *Callender* (1) was based on examination of freeze dried as well as formalin fixed material

RESULTS

Clinical data and microscopic findings are summarized in Table 1

As can be seen, four of the eleven melanomas displayed cells with a specific fluorescence, whereas the remaining seven contained no such cells Under the conditions of observation the fluorescence was in the yellow-green range and localized to the cytoplasm the nuclei being dark In two of the four tumours most of the cells were strongly fluorescent (Fig 1), whereas in the other two the fluorescent cells

This investigation was supported by a grant from the *Swedish Cancer Society*

TABLE 1

Case	Sex	Age	Localization	*Pigment* content	Type according to *Callender*	Fluorescence
1	♀	75	*Ciliary body*	High	B, scattered areas with epitheloid cells	+(+)
2	♀	67	Choroid	Varying	*A, some B*	0
3	♀	68	Choroid and *ciliary* body	Slight	B, very few epitheloid cells	0
4	♀	57	Choroid		B, many epitheloid cells	0
5	♂	55	Iris	None	A	0
6	♂	73	*Choroid*	None	B and epitheloid cells	0
7	♀	40	Choroid and ciliary body	Varying	B and scattered epitheloid cells	+++
8	♀	54		*Varying*	A and B, scattered areas with epitheloid cells	+(+) or 0
9	♀	65	Choroid	Varying	B	0
10	♀	37	Choroid and ciliary body	Varying	*Epitheloid*	+++
11	♂	40	Choriod and ciliary body	Slight	B	0

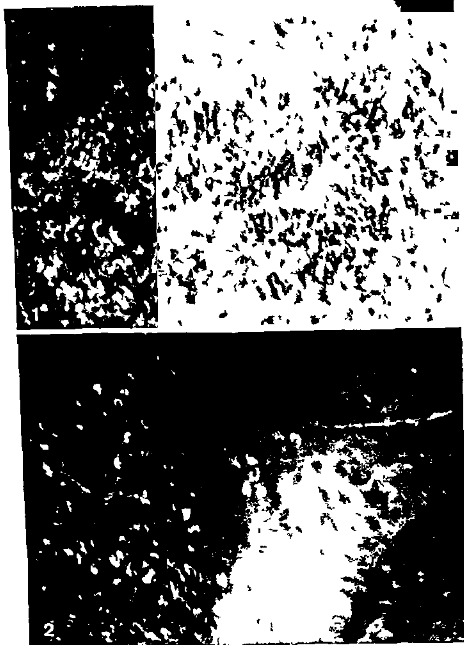


Fig. 1. Human ocular malignant m[illegible]l[illegible] m [illegible] M[illegible] f the cells shows a m[illegible]lerate [illegible] r strong specific fluore[illegible]cence.

Fig. 2. Human [illegible]cular mal[illegible]gnant m[illegible]l[illegible] me cells in a part [illegible]f th[illegible]e t[illegible]m[illegible]ur sh[illegible]w any specif[illegible]c fluore[illegible]n[illegible]



Fig 3
Human ocular malignant melanoma The cells with a specific fluorescence are polymorph with a dark nucleus and varying intensity of the cytoplasmic fluorescence × 530

were few and had a lower fluorescence intensity (Fig 2) In the two tumours with a smaller number of fluorescent cells, regions containing non-fluorescent cells were irregularly distributed throughout the tumour Usually the degree of pigmentation and the fluorescence seemed to vary independently of each other However, to a certain degree the fluorescence could be masked by a dense accumulation of pigment The morphology of the fluorescent cells varied with intermingled rounded, polygonal, spindle-shaped or dendritic cells (Fig 3) The fluorescence of the cells varied from none at all to very intense but did not seem to be correlated with any special cell type However the cells with the most intense fluorescence were often rounded and large

Comparison with routine stained sections showed that cells with the same appearance could be either fluorescent or non fluorescent

In one patient with strongly fluorescent cells in the tumour, the branched melanocytes in the anterior layer of a part of the iris displayed yellowish fluorescence throughout the cytoplasm The intensity of the fluorescence here varied between different cells and was generally highest in the most superficial cells

DISCUSSION

Only four of the eleven malignant melanomas of the eye displayed fluorescent cells. In the skin all malignant melanomas studied contained fluorescent cells (6). This difference in the fluorescence characteristics of the tumours of the skin and the eye could not be predicted from earlier knowledge of these tumours, and must be considered significant.

There is evidence that tyrosinase activity is correlated with the fluorescence intensity of melanin producing cells (6). Thus, highly active melanin producing cells of the skin demonstrate tyrosinase activity (9, 10) and strong fluorescence (6) whereas no tyrosinase activity (13) or specific fluorescence has been found in the melanin containing cells of the adult eye (2).

The total absence of fluorescence in many tumours and the absence of fluorescence in many cells in some of the melanomas containing fluorescent cells shows that fluorescence is not firmly bound to the neoplastic growth of the melanin-producing cells. It seems possible that the melanin formation in some ocular melanomas need not result in high concentrations of dopa within the melanin synthesizing cell. Differences in fluorescence between cells in one and the same melanoma might be explained by the cells being different in melanin synthesizing capacity but might perhaps also be a sign of different timing of melanin synthesis in the cells.

The fluorescence of the cells could not be correlated with the pathological subdivision of ocular melanomas according to *Callender* (1) nor with the melanin content. The strongest fluorescence observed seemed to be in large epitheloid cells and in highly branched cells. In melanomas of mixed type the lack of correlation between fluorescence and the morphology of the cells was particularly evident.

In one patient with an ocular melanoma containing fluorescent tumour cells, fluorescing melanocytes were observed in a part of the iris. It seems possible that the tyrosinase of the observed iridic melanocytes was activated by the malignant growth of the eye. Activation of iris freckles has been observed in connection with ocular melanomas (15).

SUMMARY

Eleven ocular melanomas were studied with a specific fluorescence method demonstrating catachol[illegible]ines [illegible]droxytryptamine and their immediate precursors dopa an[illegible] tryptophan. In four tumours fluorescent cells were found [illegible]rce of fluorescence did not correlate with the cell type [illegible] haematoxylin eosin st[illegible]d sections. The absence of fluo[illegible] many ocular melan[illegible]s in contrast to the presence of s[illegible]rescence in cutane[illegible]lanomas

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Acta path et microbiol scandinav 69, 185–191, 1967

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EVOLUTIONARY TRENDS OF ANEUPLOID BLOOD CULTURE CELL POPULATIONS DURING PROGRESSION AND TREATMENT OF CHRONIC MYELOGENOUS LEUKAEMIA

By

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Received 20 vii 66

It appears probable from previous cytogenetic investigations that, within the Ph^{1}-positive cell populations, selection of certain cell categories occurs during progression and treatment with cytostatics of chronic myelogenous leukaemia Both during progression of the disease and during therapy, hyperdiploid cells appear to be favoured at the expense of other Ph^{1}-positive cells (*Pedersen* 1966a) The high prevalences of hyperdiploid, Ph^{1}-positive cells in the late phase of the disease and during treatment with cytostatics may, however, be due to a few, well-represented cell clones If this is the case, the clones concerned are possibly favoured but not necessarily hyperdiploid karyotypes as a whole If the selective pressure of the leukaemic *in vivo* environment, including cytostatics, electively favours the proliferation and/or survival of hyperdiploid cells, increased prevalence of various hyperdiploid karyotype variants would be anticipated in the late phase of the disease and during treatment with cytostatics

The object of the present work is to illustrate this question by comparing the number of aneuploid karyotype variants which have developed independently of one another in the phase and treatment groups of a blood culture material

In order to assess the extent to which artificial factors, including cytostatics, are *eo ipso* responsible for the aneuploid state of the population, the Ph^{1}-positive cells will be compared with a Ph^{1} negative population which originates from the same blood culture material as the Ph^{1}-positive cells

MATERIAL AND METHODS

The material consists of Ph^{1} positive [illegible] blood culture cells from patients with clinically and haematologically [illegible] myelogenous leukaemia [illegible]

Supported by a scholarship from the [illegible] Memorial Trust

were submitted to investigation on one to seven different occasions in the course of the disease Further information concerning the collection of the material its nature and extent the technique of the *in vitro* culture harvesting of the cultured material and preparation of the specimens for chromosome analysis is given elsewhere (*Pedersen* 1966 a) where the principles for selection of the mitoses for analysis and the method of analysis are also mentioned

The aneuploid cells are subdivided according to the chromosome counts into the groups <45 45 46U and >46 The 46U group consists of unbalanced diploid cells i e cells with 46 chromosomes but aneuploid karyotypes (*Levan & Müntzing* 1963) The Ph¹ negative cell material originates as mentioned from the same cultures as the Ph¹ positive cells and was therefore, cultured harvested and prepared in the same manner and exposed to the same cytostatic treatment as the Ph¹ positive cells Further Ph positive and negative cells were analysed at random The two cell populations were finally subdivided into groups according to the same principles Cells which could not be classified with certainty as Ph¹ positive or Ph¹ negative were not included in the present material

In order to illustrate the structure of the aneuploid cell population during the progression of the disease the material is subdivided into three groups

1 Cultures from untreated patients all of whom were newly diagnosed at the time of investigation (untreated phase)

2 Cultures from treated patients in the early phase of the disease i e patients who at the time of investigation had received cytostatic treatment for a total of less than 150 days

3 Cultures from patients in the late phase of the disease i e patients who at the time of investigation had received cytostatic treatment for 150 days or more

In order to illustrate the possible effects of treatment the material was further subdivided into two treatment groups

1 Cultures from patients who at the time of investigation were receiving cytostatic treatment or whose last treatment series was concluded less than ten days previously (group T 10—)

2 Cultures from patients whose last treatment series was concluded more than ten days previously (group T 10+)

In each of the groups in the material only one representative from each aneuploid karyotype from each patient is included The prevalences of aneuploid cells calculated on this basis thus express the frequencies with which the aneuploid karyotype variants have developed independently of one another

The phase and treatment groups will be compared in the following manner untreated/early phase early/late phase and groups T 10+/T 10— In each pair of groups compared cultures are only included from patients represented in both groups In the early and late phase groups cultures from the same patient belong to the same treatment group In the treatment groups cultures from the same patient belong to the same phase group The relevant material includes 65 cultures from 18 patients

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RESULTS

The untreated and the early treated phases of the disease do not show significantly different prevalence of members in the various chromosome count groups (Table 1), neither in the Ph¹-positive nor the Ph¹ negative cell population The late phase of the disease, on the other hand contains significantly more Ph¹-positive cells with hyperdiploid karyotypes than the early phase (Table 2) Further the late phase shows a tendency to the lowest frequency of 45 cells and highest prevalence of 46 U cells In the Ph¹ negative population, the two phases do not show any definitely different distribution in the aneuploid

TABLE 1

Chromosome Count Distributions in Ph¹ Positive and Ph¹ Negative Cells
Untreated versus Early Treated Phase of Disease

Chromosome Count	Ph¹ Positive Cells: Phase of Disease		P	Ph¹-Negative Cells: Phase of Disease		P
	Untreated	Early		Untreated	Early	
<45	13	8	>0.05	5	5	>0.05
45	17	9	>0.05	3	19	>0.05
46U	5	4	>0.05	–	5	>0.05
>46	1	5	>0.05	1	7	>0.05
Total	36	26	>0.05	9	36	>0.05
No of Cultures	8	13		7	10	
Analysed Cells	245	145		110	342	

TABLE 2

Chromosome Count Distributions in Ph¹ Positive and Ph¹ Negative Cells
Early versus Late Phase of Disease

Chromosome Count	Ph¹ Positive Cells: Phase of Disease		P	Ph¹-Negative Cells: Phase of Disease		P
	Early	Late		Early	Late	
<45	5	7	>0.05	2	2	>0.05
45	11	8	>0.05	7	6	>0.05
46U	5	18	>0.05	8	5	>0.05
>46	6	43	<0.0005	2	–	>0.05
Total	27	76	0.010–0.025	19	13	>0.05
No of Cultures	9	8		5	3	
Analysed Cells	169	287		185	66	

TABLE 3

Chromosome Count Distributions in Ph¹-Positive and Ph¹ Negative Cells
Treatment Group T 10+ versus T 10—

Chromosome Count	Ph¹ Positive Cells: Treatment Groups		P	Ph¹ Negative Cells: Treatment Groups		P
	T 10+	T 10—		T 10+	T 10—	
<45	6	3	>0.05	7	4	>0.05
45	15	5	[illegible]0.05	16	12	>0.05
46U	12	10	[illegible].05	16	20	>0.05
>46	14	23	[illegible]0.010	9	3	>0.05
Total	47	41	[illegible]	48	39	[illegible]0.05
No of Cultures	16	1[illegible]		14	14	
Analysed Cells	301	1[illegible]		339	332	

chromosome count groups In Table 3, the two treatment groups are compared The prevalence of hyperdiploid, Ph^1-positive cells is significantly higher in group T 10— than in group T 10+ The frequency of Ph^1 positive 45 cells is highest in the group T 10+ but the difference from group T 10—, however, is not significant The Ph^1 negative population does not show any definitely different prevalences

TABLE 4

Chromosome Count Distributions in Ph^1 Positive versus Ph^1 Negative Cells of Untreated Early and Late Phases of Disease

Chromosome Count	Phase of Disease								
	Untreated			Early			Late		
	Ph^1 Pos	Ph^1 Neg	P	Ph^1 Pos	Ph^1 Neg	P	Ph^1 Pos	Ph^1 Neg	P
<45	13	5	>0.05	5	2	>0.05	7	2	>0.05
45	17	3	>0.05	11	7	>0.05	8	6	0.03
46L	5		>0.05	5	8	>0.05	18	5	>0.05
>46	1	1	>0.05	6	2	>0.05	43		0.001 0.005
Total	36	9	>0.05	27	19	>0.05	76	13	>0.05
No of Cultures	8	7		9	5		8	3	
Analysed Cells	245	110		169	185		287	66	

TABLE 5

Chromosome Count Distributions in Ph^1 Positive versus Ph^1 Negative Cells of Treatments Groups T 10+ and T 10—

Chromosome Count	Treatment Groups					
	T 10+			T 10—		
	Ph^1 Pos	Ph^1 Neg	P	Ph^1 Pos	Ph^1 Neg	P
<45	6	7	>0.05	3	4	>0.05
45	15	16	>0.05	5	12	>0.05
46L	12	16	>0.05	10	20	>0.05
>46	14	9	>0.05	23	3	<0.0005
Total	47	48	>0.05	41	39	0.005 0.010
No of Cultures	16	14		10	14	
Analysed Cells	301	339		195	372	

In Table 4 the distributions of the Ph^1 positive and negative cells *in the four chromosome count groups in the three phase groups of the* material are compared Neither the untreated nor the early treated phases show definite differences in prevalence between the two populations In the late phase, *hyperdiploid cells occur significantly more* frequently in the Ph^1 positive than in the Ph^1 negative population In

Table 5 the two populations are compared in each treatment group *per se* While group T 10+ does not show any definite difference, the Ph¹-positive prevalence of hyperdiploid cells is significantly higher than the Ph¹ negative in group T 10—

DISCUSSION

The cytogenetic basic material from which the present material originates is particularly heterogeneous Attempts were made to make the phase and treatment groups comparable in the following ways

1 It cannot be excluded in advance that the tendency to form aneuploid cells is an individual feature, i e independent of the progression and treatment of the chronic myelogenous leukaemia If the patients included in the phase and treatment groups compared are not identical, the possibility must be borne in mind that the differences between these groups may be due to the patients in the groups rather than the phase of the disease and the situation as regards treatment An attempt was made to eliminate this possible source of error by only including patients who were represented by one or more cultures in both groups in each pair of groups compared Further, it was required that corresponding cultures in the early and late phase groups must belong to the same treatment group and that the corresponding cultures in the treatment groups T 10+ and T 10— must originate from the same phase of the disease It is thus possible to include both T 10— and T 10 + cultures in both the early and the late phase groups and to include cultures from both the early and late phase groups in both treatment groups T 10+ and T 10— It should be noted, however that the early phase group which is compared with cultures from untreated patients consists of both T 10— and T 10+ cultures

2 By including only one representative of each aneuploid karyotype in each of the groups in the material, aneuploid clones comprising numerous cells are prevented from dominating the aneuploid cell populations of the corresponding phase and treatment groups

These attempts to work with reasonably comparable groups have, however, resulted in considerable reduction of the extent of the relevant material

The results of the present investigation are by and large, in agreement with a previous investigation of the same cytogenetic basic material (*Pedersen* 1966a) This investigation together with the present investigation shows that during progression of the disease, increasing prevalence of hyperdiploid, Ph¹-positive cells occurs and that these cells include an increasing number of karyotype variants Further the prevalence of hyperdiploid Ph¹-positive cells and the number of hyperdiploid karyotypes involved are seen to be greater in the treatment group T 10— than in group T 10+ [illegible] comparing the cytogenetic profiles of the treatment groups it must, however, be borne in mind that

the average duration of the cytostatic treatment was somewhat longer in group T 10— (235 5 days per Ph¹-positive cell) than in group T 10+ (183 6 days per Ph¹-positive cell). It does not appear probable that this difference can explain the divergent cytogenetic conditions in the two groups

The prevalence of Ph¹ positive 45 cells was previously found to decrease during progression of the disease and during treatment with cytostatics, while the frequency of Ph¹-positive 46 U cells shows an indefinite relationship to progression of the disease and decreases during treatment (*Pedersen* 1966a) In the present material, the two categories of cells show tendencies which are in agreement with this but the differences are not significant

In contrast to the Ph¹ positive cells, the composition of the aneuploid Ph¹-negative cell population does not reveal any definite dependence upon progression or treatment of the disease Had cytostatic treatment been a material cause of development of aneuploid cell variants, it would be anticipated that the Ph¹-positive and -negative cell populations would show corresponding development during progression and treatment As this is not the case, it may be concluded that cytostatics *eo ipso* do not cause aneuploidy in blood culture cells from patients with chronic myelogenous leukaemia to any appreciable extent

The high prevalence of hyperdiploid, Ph¹-positive karyotypes in the late phase group and in treatment group T 10— cannot be due to *in vitro* selection of hyperdiploid cells in the cultures which originate from blood samples with sporadic occurrence of such cells, as a comparison of the cytogenetic conditions in uncultured bone-marrow biopsies from patients with chronic myelogenous leukaemia and blood cultures which originate from the same patients and the same time did not show signs of selection of hyperdiploid cells (*Pedersen* 1966b)

The present observations are, however, consistent with the assumption that selection of hyperdiploid, Ph¹-positive cells occurs *in vivo* in the late phase of chronic myelogenous leukaemia This selection is probably due not only to weakening of the resistence of the organism towards cytogenetically abnormal cells as this would presumably favour all categories of aneuploid cells, Ph¹-positive and Ph¹-negative Bearing in mind that the highest prevalences of hyperdiploid Ph¹-positive cells (*Pedersen* 1966a) and karyotype variants (the present investigation) originate from cultures from patients receiving intensive cytostatic treatment, it appears reasonable to presume that the cytostatics employed are, to a great extent, responsible for selection of Ph¹-positive cells with hyperdiploid karyotypes because these are, somehow or other, less sensitive to the treatment than other cells

The present investigation cannot illustrate the question whether hyperdiploid chromosome counts *eo ipso* are associated with selective advantages or whether such advantages are associated with definite karyotypes

SUMMARY

In a previous investigation, the prevalence of hyperdiploid, Ph¹-positive cells in a blood culture material from 29 patients with chronic myelogenous leukaemia investigated cytogenetically was found to be significantly higher in the late phase of the disease than in its early phase and higher in cultures from patients receiving treatment with or recently treated with cytostatics than in cultures from patients in whom the last period of treatment had been concluded longer ago (*Pedersen* 1966a) The object of the present work was, in the same cytogenetic basic material, to investigate whether the number of hyperdiploid karyotype variants involved increases during the progression and treatment of the disease For this reason, only one representative of each aneuploid karyotype in each of the phase and treatment groups was included in the material from each patient In order to increase the comparability of the groups, only patients were included in each pair of groups compared who were represented by one or more cultures in both groups of the pair

While cultures from untreated patients and treated patients in the early phase of the disease did not show definite differences, the prevalence of hyperdiploid, Ph¹ positive karyotype variants was significantly higher in the late phase of the disease than in its early phase and higher in cell populations from patients receiving cytostatics at the time of investigation or who had concluded this treatment a few days previously than in the population from patients whose last series of treatment had been concluded longer ago The remaining aneuploid chromosome count groups did not show any significant difference between the phase and treatment groups compared

As a comparable Ph¹ negative population did not show differences associated with progression and treatment of the disease, it is concluded that the differences observed in the Ph¹ positive cell populations cannot be due to direct karyotype damage caused by cytostatics to any appreciable extent, as the Ph¹-positive and -negative populations have been subjected to cytostatics to much the same degree The observations seem to indicate that the spectrum of viable hyperdiploid, Ph¹-positive karyotypes is broader in the late than in the early phase of the disease and broader during cytostatic treatment than in treatment free periods An important reason for this state of affairs may be that cells with hyperdiploid, Ph¹-positive karyotypes are relatively more resistent to the cytostatics employed than other cells

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Acta path et microbiol scandinav 69 192–204, 1967

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CYTOGENETIC STRUCTURE OF ANEUPLOID BLOOD CULTURE CELL POPULATIONS DURING PROGRESSION AND TREATMENT OF CHRONIC MYELOGENOUS LEUKAEMIA

By

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Received 20 xii 66

Previous investigations of Ph^1-positive blood culture cells from patients with chronic myelogenous leukaemia suggest that selection of cells with certain chromosome counts occurs at the expense of other cells during progression of the disease Among other features, the cell populations from patients in the late phase of the disease appear to contain significantly higher prevalences of hyperdiploid, Ph^1-positive cells than populations from patients in the early phase of the disease (*Pedersen* 1966a) and, similarly, the number of hyperdiploid, Ph^1-positive clones was found to be significantly higher in the late than in the early phase (*Pedersen* 1967) In addition, the frequency of hyperdiploid, Ph^1-positive cells was found to be definitely higher in blood cultures from patients who were receiving treatment with cytostatics at the time of investigation or in whom the last series of treatment had been concluded immediately prior to the cytogenetic investigation than in blood cultures from patients in whom treatment had been concluded longer ago The former treatment group contained, further, a higher prevalence of different hyperdiploid, Ph^1-positive clones than the latter group It appears possible that selection of hyperdiploid, Ph^1-positive cells, on a cytogenetic basis, depends upon their karyotypes rather than their chromosome counts In the present investigation, an attempt will be made to illustrate this question by comparing the karyotype patterns in Ph^1-positive, aneuploid karyotypes from different phase and treatment groups The investigation was carried out on the same cytogenetic material as formed the basis for the works mentioned above The Ph^1-positive cell material was compared with a comparable Ph^1-negative material in an attempt to estimate the possible significance of cytostatic drugs and technical factors for the Ph^1-positive aneuploidy

Supported by a scholarship from the *Lady Tata Memorial Trust*

MATERIAL AND METHODS

The material consists of Ph[1] positive and negative blood culture cells from patients with clinically and haematologically typical chronic myelogenous leukaem a who were investigated on 1 7 d fferent occasions during the course of the disease Further information concerning collection of the material its nature and extent the tech n que of the *in vitro* culture harvesting of the blood cultures and preparation of the specimens is given elsewhere (*Pedersen* 1966 a) where the principles of selection of the mitoses in the material for analysis and the methods of analysis are described in more detail

In many cells it is not possible to differentiate Y from the other small acrocentric chromosomes with certainty This is particularly true in the case of Ph[1] positive cells where partially despiralized chromatin substance makes distinction difficult For this reason group (21 22) and Y are treated as one group in the present work

The object of the investigation is to determine the frequency with which members of the various chromosome groups are lost and acquired during the abnormal cell divisions which are the cause of the aneuploid clones in the material As members of an aneuploid clone are probably all the result of one or the same abnormal cell divisions only one representative of each aneuploid karyotype was included in each of the phase and treatment groups in the material of aneuploid cells the chromo- s me d stribution of wh ch has l een publ shed elsewhere (*Pedersen* 1967)

An attempt was made to illustrate the structure of the aneuploid cell population during progression of the disease by comparison of cells from three phases of the disease

1 Cultures from untreated patients *All of the patients in this group were newly* diagnosed

2 Cultures from patients in the early phase of the disease i e from patients who had been treated with cytostatics for less than a total of 150 days

3 Cultures from patients in the late phase of the disease i e from patients who

was in addi

eceiving cyto- static treatment or in whom the last series of treatment was concluded less than ten days previously (group T 10—)

2 Cultures from patients whose last series of treatment had been concluded more than ten days previously (group T 10+)

Patients were only included in the pairs of phase and treatment groups com pared if they were represented by one or more cultures in both of the groups of the pair concerned In add tion early and late phase cultures from the same patient originate from the same treatment group S milarly in the treatment groups cultures from the same patient belong to the same phase group Desp te this the average durati n of the cytostatic treatment was somewhat longer in group T 10— (235 5 days per Ph[1] positive cell) than in group T 10+ (183 6 days per Ph[1] pos tive cell) After sorting out in th s manner the relevant material comprises 65 cultures from 18 patients

The Ph[1] negative cell material originates from the same cultures as the Ph[1] positive cells Both cell populations were therefore grown harvested and prepared in the same manner and were expose l to cytostatic treatment to the same or more or less the same

material th s d es not contain c . . . h ut l h wh ch lack small acrocentr c chr mos mes the prevalence of a . . . 21 . . . meml ers in Ph[1] negative cell could not be calculated

The prevalence of extra chr n . . . group is the number . . . chromos mes d v led l y the numb r f t . . . bers ol served in the . . . the extra chromos mes divided b . . . er of chr m somes ob . . . nus the extra chrom somes The pre . . . n chromosomes . . .

TABLE 1

Prevalences of Extra Chromosomes in Ph^1 Positive and Ph^1 Negative Cells
Untreated versus Early Treated Phase of Disease

Chromosome Groups	Ph^1 Pos Cells Phase of Disease: Untreated Extra/Others	Ph^1 Pos Cells Phase of Disease: Early Treated Extra/Others	P	Ph^1 Neg Cells Phase of Disease: Untreated Extra/Others	Ph^1 Neg Cells Phase of Disease: Early, Treated Extra/Others	P
21 22 Y	6/1136	5/644	>0 05	0/503	3/1657	>0 05
19 20	2/974	1/579	>0 05	0/438	3/1358	>0 05
17 18	0/977	0/576	>0 05	0/439	3/1369	>0 05
16	0/484	0/289	>0 05	0/218	1/677	>0 05
13 15	0/1463	2/986	>0 05	0/657	1/2040	>0 05
6 X 12	1/3749	2/2246	>0 05	1/1692	4/5153	>0 05
1 5	0/2449	0/1449	>0 05	0/1100	2/3404	>0 05
Total	9/11232	10/6769	>0 05	1/5047	17/15658	>0 05
No of Cultures	8	13		7	10	

TABLE 2

Prevalences of Extra Chromosomes in Ph¹ Positive and Ph¹ Negative Cells
Early versus Late Phase of Disease

Chromosome Groups	Ph¹ Pos Cells Phase of Disease Early Extra/Others	Ph¹ Pos Cells Phase of Disease Late Extra/Others	P	Ph¹ Neg Cells Phase of Disease Early Extra/Others	Ph¹ Neg Cells Phase of Disease Late Extra/Others	P
21 - Y	3/789	52/1419	<0.0005	0/779	0/273	>0.05
19 20	3/671	13/1141	>0.05	0/738	0/262	>0.05
17 18	1/675	1/1036	>0.05	2/738	3/202	>0.05
16	1/337	0/571	>0.05	3/368	0/131	>0.05
13 15	1/1009	8/1713	>0.05	0/1103	0/396	>0.05
6 X 12	4/2578	150/4304	<0.0005	4/2914	2/1042	>0.05
1 5	0/1689	24/2864	<0.0005	1/1846	1/657	>0.05
Total	13/7748	248/13108	<0.0005	16/8486	6/3023	>0.05
No of Cultures	9	8		5	3	

calculated by dividing the number of absent chromosomes by the number of members observed in the chromosome group concerned Cells with 43 or fewer chromosomes are not included in these calculations

The basis for the calculations of prevalence of extra (21 22-Y) members in the Ph[1] negative cells differed slightly from the basis for the other calculations As the number of absent (21 22 Y) members in the Ph[1] negative cells is not known and therefore cannot be included in the calculation, the number of small acrocentric chromosomes recorded is slightly too high in relation to the number of extra members The prevalences of extra (21-22-Y) members calculated in Ph[1] negative cells is, therefore, slightly lower than the actual number

The frequencies of extra and absent chromosomes in the phase and treatment groups of the material were compared by means of χ^2 tests Where the expected numbers were less than 5, the probability for the distribution observed in the groups compared was found by means of tables of binomial probability distributions

RESULTS

The frequency of extra members in each individual chromosome group is compared in Table 1 in cultures from the untreated and the early treated phases of the disease Neither the Ph[1]-positive nor -negative population show significant differences In the late, Ph[1]-positive phase group, extra (21–22 Y), (6–X–12) and (1–5) were significantly more frequent than in the corresponding early phase group (Table 2) The extra (1–5) members originate from two cultures from two patients All 24 extra chromosomes belong to chromosome pair no 1 The Ph[1]-negative population does not show any definite difference between the two phase groups Table 3 shows that extra (6–X–12) occur more frequently in the Ph[1]-positive group T 10— than the group T 10+, whereas the Ph[1] negative cells do not show significant differences between the two treatment groups

Table 4 compares the prevalences of absent members within the individual chromosome groups in cultures from the untreated and early treated phase groups No significant differences were found in either the Ph[1]-positive or the Ph[1]-negative populations Members of the (17 18) group were absent in Ph[1]-positive cells more frequently in the late phase of the disease than the early phase (Table 5) The Ph[1]-negative population does not show any significant difference between the two phase groups Neither in the Ph[1]-positive nor in the Ph[1]-negative populations were significant differences in prevalence of absent chromosomes observed between the two treatment groups (Table 6) but the Ph[1]-positive cells, however, showed a tendency to the greatest prevalence of absent (17–18) members in group T 10— ($0.05 < P < 0.10$)

Comparison of Ph[1]-positive and Ph[1]-negative prevalences of extra and absent chromosomes in each individual chromosome group in the phase and treatment groups of the material gives results which, by and large, are in agreement with the above Cultures from the untreated and early treated phases do not show definite differences In the late phase, extra (21–22–Y), extra (6–X–12) and absent (17–18) members were significantly most frequent in the Ph[1]-positive population (0 001

TABLE 3

Prevalences of Extra Chromosomes in Ph¹ Positive and Ph¹ Negative Cells Treatment Group T 10+ versus T 10—

Chromosome Groups	Ph¹ Pos Cells Treatment Groups T 10+ Extra/Others	T 10— Extra/Others	P	Ph¹-Neg Cells Treatment Groups T 10+ Extra/Others	T 10— Extra/Others	P
[illegible]	0/1393	5/953	>0 05	0/1515	0/1619	>0 05
[illegible]	6/1200	5/779	>0 05	3/1369	2/1301	>0 05
[illegible]	0/1193	1/765	>0 05	2/1367	1/1302	>0 05
[illegible]	3/[illegible]	0/787	>0 05	1/676	0/652	>0 05
[illegible]	1/1797	4/1167	>0 05	3/2029	2/1957	>0 05
[illegible]	11/4612	19/2930	<0 0005	13/5191	10/4912	>0 05
[illegible]	0/3008	0/1348	>0 05	0/3395	3/3261	>0 05
Total	[illegible]/13802	64/8035	<0 0005	30/15624	24/15008	>0 05
No of Cultures	16	10		14	14	

TABLE 4

Prevalences of Absent Chromosomes in Ph^1 Positive and Ph^1 Negative Cells Untreated versus Early Treated Phase of Disease

Chromosome Groups	Ph^1 Pos Cells Phase of Disease Untreated Absent/Others	Ph^1 Pos Cells Phase of Disease Early, Treated Absent/Others	P	Ph^1 Neg Cells Phase of Disease Untreated Absent/Others	Ph^1 Neg Cells Phase of Disease Early, Treated Absent/Others	P
21 22 Y	5/1142	6/649	>0 05	•	•	•
19 20	6/976	1/580	>0 05	2/428	6/1361	>0 05
17 18	3/977	4/576	>0 05	1/439	3/1372	>0 05
16	6/484	1/289	>0 05	2/218	5/678	>0 05
13 15	7/1403	4/988	>0 05	3/657	0/2041	>0 05
6 X 12	10/3750	4/2248	>0 05	5/1693	10/5157	>0 05
1 5	1/2443	1/1449	>0 05	0/1100	6/3406	>0 05
Total	38/11241	21/6779	>0 05	13/4545	36/14015	>0 05
No of Cultures	8	13		7	10	

TABLE 5

Prevalences of Absent Chromosomes in Ph¹ Positive and Ph¹ Negative Cells Early versus Late Phase of Disease

Chromosome Groups	Ph¹ Pos Cells, Phase of Disease: Early Absent/Others	Late Absent/Others	P	Ph¹-Neg Cells, Phase of Disease: Early Absent/Others	Late Absent/Others	P
21 22 Y	7/792	7/1471	>0 05	•	•	•
19 20	5/674	7/2154	>0 05	2/738	2/262	>0 05
17 18	1/676	52/1097	<0 0005	2/740	2/265	>0 05
16	1/338	3/571	>0 05	2/371	1/131	>0 05
	5/1010	9/1721	>0 05	7/1103	0/396	>0 05
C X 2	6/2582	10/4454	>0 05	7/2918	5/1044	>0 05
1 3	1/1089	6/2888	>0 05	4/1847	3/658	>0 05
Total	26/7761	94/13356	<0 0005	24/7717	13/2756	>0 05
No of Cultures	9	8		5	7	

TABLE 6

Prevalences of Absent Chromosomes in Ph1 Positive and Ph1 Negative Cells Treatment Group T 10+ versus T 10—

Chromosome Groups	Ph1 Pos Cells Treatment Groups T 10+ Absent/Others	Ph1 Pos Cells Treatment Groups T 10— Absent/Others	P	Ph1-Neg Cells Treatment Groups T 10+ Absent/Others	Ph1-Neg Cells Treatment Groups T 10— Absent/Others	P
21-22 Y	9/1399	10/958	>0.05	*	*	*
19-20	4/1206	1/784	>0.05	3/1372	5/1305	>0.05
17-18	11/1193	15/766	>0.05	5/1369	6/1303	>0.05
16	5/602	3/387	>0.05	4/679	2/652	>0.05
13-15	9/1800	3/1171	>0.05	11/2032	5/1959	>0.05
6-X-12	6/4643	1/2985	>0.05	12/5206	9/4922	>0.05
1-5	2/3008	2/1948	>0.05	5/3395	7/3266	>0.05
Total	46/13851	35/8999	>0.05	40/14053	34/13407	>0.05
No. of Cultures	16	16		14	14	

< P < 0 005 P < 0 0005 0 005 < P < 0 010 respectively) In addition the Ph^1 positive population contains the highest prevalence of extra (6–X 12) members in both groups T 10+ and T 10— (0 001 < P < 0 005 and P < 0 0005 respectively) Finally the Ph^1 positive prevalence of absent (17 18) members in group T 10— is higher than the Ph^1 negative (0 001 < P < 0 005)

DISCUSSION

The phase and treatment groups compared are considered to be reasonably comparable This point has been discussed elsewhere (*Pedersen* 1966b)

As mentioned previously in the present investigation no differentiation was undertaken between (21 22) and Y in the group of small acrocentric chromosomes As this group represents a considerable fraction of the extra chromosomes in the material it would be of interest to know whether the extra members were (21 22) or Y Numerous aneuploid Ph^1 positive clones with extra small acrocentric chromosomes have been described in the literature (*Adams et al* 1961 *Court Brown & Tough* 1963 *Hammouda* 1963 *Hammouda et al* 1964 *Kemp et al* 1964 *Lejeune et al* 1965 *de Grouchy et al* 1965a and b *Kiossoglou et al* 1965 *Dougan & Woodliff* 1965) None of these clones contained extra Y chromosomes The present material contains in the early phase three and in the late phase 52 extra small acrocentric chromosomes (Table 2) Of these 55 chromosomes 48 originate from cells with two Ph^1 Despite the indefinite character of the chromatin in many of the cells the extra members with quite a great degree of certainty were found to be (21 22) in all 38 cells representing the 55 chromosomes Extra Y members do not appear to be included in the composition of the aneuploid Ph^1 positive cells in the material

Analysis of the distribution of the chromosome counts in the aneuploid cells of the material revealed that in the Ph^1 negative population no dependence upon the total duration or the situation as regards treatment at the time of investigation was present (*Pedersen* 1967) The results presented here show that the pattern of extra and absent chromosomes in the aneuploid Ph^1 negative population similarly is uninfluenced by these factors If cytostatic and/or technical factors were of decisive or material significance as aneuploidy producing factors the Ph^1 positive and the Ph[illegible] negative populations would have reflected this effect to a more or [illegible] uniform extent As the prevalences of extra and absent chromosom[illegible] r muc[illegible] higher and differently distributed in the chromosome [illegible] n [illegible] Ph^1 positive than in the Ph^1 negative population the P[illegible] t [illegible] n uploidy must have other causes

The karyotype pattern of the [illegible] Ph^1 positive cell pop[illegible] n cannot be explained by *in vitr* [illegible] s blood cultures fr[illegible] patients

with chronic myelogenous leukaemia do not appear to favour Ph¹-positive cells with definite karyotypes (*Pedersen* 1966b).

The distribution of the chromosome counts in the aneuploid cells of the present material shows that cultures from patients in the late phase of the disease contain significantly higher prevalences of hyperdiploid, Ph¹-positive clones than cultures from the early phase In addition, these clones occur with greater frequency in treatment group T 10— than in group T 10+ (*Pedersen* 1967) It is apparent from the present investigation that the late phase and treatment group T 10— present higher Ph¹-positive prevalences of extra (21–22), (6–X–12), (1–5) and absent (17–18) than the other groups of the material The high prevalences of these karyotype abnormalities in the phase and treatment group which contains the greatest frequency of hyperdiploid, Ph¹-positive cells suggests that these, particularly, characterize the hyperdiploid, Ph¹-positive cell population If selection of hyperdiploid, Ph¹-positive cells was due to an abnormal chromosome count, prevalences of extra chromosomes would be anticipated to be more or less uniform in the various chromosome groups Concentration of the extra chromosomes in three out of the seven chromosome groups suggests that selection of hyperdiploid, Ph¹-positive clones in the leukaemic *in vivo* environment is due to the karyotypes of the clones and not to their chromosome counts

It should be noted that the results of the present investigation must be assessed bearing in mind the limited extent of the material The results must not be interpreted to suggest that, generally, selection of clones with extra (21–22), (6–X–12), (1–5) and absent (17–18) members occurs in patients with chronic myelogenous leukaemia It is probable that other materials can demonstrate selection of clones with other karyotypes For example the Ph¹-positive cells of this material contain only two extra (17–18) members (Table 2) as compared with 53 absent members of this group (Table 5) which gives the impression that Ph¹-positive clones with extra (17 18) members are more poorly adapted to the leukaemic *in vivo* environment than clones in which (17 18) members are absent The numerous descriptions in the literature of Ph¹-positive, aneuploid clones with extra (17–18) members (*Kemp et al* 1963, *Court Brown & Tough* 1963, *Hammouda et al* 1964 *Lejeune et al* 1965, *de Grouchy et al* 1965 a and b) and few descriptions of clones in which members of this group are absent (*Court Brown & Tough* 1963 *Hammouda* 1963) give however, a different impression of the relative frequency of extra and absent (17 18) members in aneuploid, Ph¹-positive clones The clones described in the literature however, as in the present results, are dominated by karyotypes with extra (21 22 Ph¹) (*Adams et al* 1961 *Court Brown & Tough* 1963 *Hammouda* 1963, *Jung et al* 1963, *Reisman & Trujillo* 1963, *Trujillo & Ohno* 1963 *Hammouda et al* 1964 *Kemp et al* 1964 *Lejeune et al* 1965, *de Grouchy et al* 1965 a and b, *Kiossoglou et al* 1965, *Dougan &*

Woodliff 1965, *Erkman et al* 1966) and extra (6–X 12) members (*Ford & Clarke* 1963, *Court Brown & Tough* 1963, *Kemp et al* 1963 *Levan et al* 1963 *Hammouda et al* 1964, *Goh et al* 1964 de *Grouchy et al* 1965 a and b *Lejeune et al* 1965, *Erkman et al* 1966)

SUMMARY

In a cell material from blood cultures from patients with chronic myelogenous leukaemia investigated cytogenetically and grouped according to phase of the disease and situation as regards treatment at the time of investigation evidence was found previously that selection of hyperdiploid, Ph[1] positive cells occurs during progression and treatment of the disease (*Pedersen* 1967) In the present investigation, the patterns of extra and absent chromosomes of these groups are compared to illustrate the question of whether the selection mentioned above occurs on the basis of the chromosome counts of the clones or is dependent of their karyotypes

Each pair of phase and treatment groups in the material includes only patients represented by one or more cultures in both of the groups of the pair The material totals 65 cultures from 18 patients Only one representative of each aneuploid karyotype from each patient is included in each group of the material The numbers of extra and absent members are compared with the number of other chromosomes observed in each chromosome group after which the prevalences are compared in the groups of the material A comparable Ph[1]-negative cell material was compared with the Ph[1] positive material in order to evaluate the influence of iatrogenic and technical factors upon the cytogenetic conditions of the Ph[1] positive population

In cultures from patients in the late phase of the disease the prevalences of extra (21 22), (6–X 12) and (1–5) members in Ph[1] positive cells were found to be significantly higher than in cultures from the early phase of the disease In cultures from patients receiving treatment with cytostatics or who had recently received such treatment at the time of investigation the prevalence of extra (6–X 12) was significantly higher, and there was a tendency towards greater frequency of absent (17–18) members than in cultures from patients in whom the last series of treatments had been concluded longer ago

As the Ph[1] negative population does not show corresponding conditions it is improbable that iatrogenic or technical factors are the cause of the development demonstr[illegible]d As selection of Ph[1] positive cells with certain karyotypes does not appear to occur in blood cultures (*Pedersen* 1966b), the result [illegible] th[illegible] [illegible]nvestigation is presumably not due to *in vitro* favouring [illegible] f Ph[illegible] [illegible]t[illegible]ve cells with the chrom[illegible]some abnormalities mention[illegible] [illegible]out [illegible]oly to *in vivo* select[illegible] [illegible]f such cells The results of th[illegible] [illegible]stigation thus sug[illegible] that selection of hyperdiploid Ph[illegible] [illegible] clones during prog[illegible]n and

treatment of chronic myelogenous leukaemia is primarily due to their karyotypes and not the high chromosome count *eo ipso*

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ON THE SPECIFICITY OF HUMAN THYROGLOBULIN AUTO ANTIBODIES

By

TORE GODAL

Received 12 vii 66

The induction of thyroglobulin auto antibodies seems to require a termination of immune tolerance towards thyroglobulin (8) In rabbits this tolerance may be terminated by injections of structurally altered homologous thyroglobulin (22)

Several other types of human auto-antibodies such as anti γ globulin factors and conglutinin seem to react with 'hidden" antigenic determinants which are unmasked in immune aggregates (9, 2)

Both these lines of observation emphasize the rôle of structural changes of auto antigens as one mechanism behind auto-antibody formation In the present investigation, attempts were made to detect human auto-antibodies against altered thyroglobulin molecules

MATERIALS AND METHODS

Antigens Thyroglobulin was prepared from normal or diseased human thyroids hog and bovine thyroids by $(NH_4)_2SO_4$ fractionation between 37 per cent and 42 per cent [illegible] fied by *Roitt &* [illegible] of purity rabbit [illegible] cent saturation [illegible] stored frozen at [illegible] but some preparations of human thyrogl bulin were lyophilized before they were stored at —20° C.

Fresh extracts of normal human thyroids were prepared from the thyroid of patients suffering from laryngeal carcinomas with ut history of thyroid disease Immediately after rem val the thyroid tissu were minced gently by scissors mixed with 2 volumes (W/V) of sterilized sal ne and shaken gently at 4° C for 4 hours The supernatant after centrifugati n [illegible] g × 30 minutes) was if not otherwise stated used the same day f r inh [illegible] f hemagglutination

Sera The sera employed could be div d [illegible] 3 groups

1 Sera from patients suffering from th [illegible] ease mainly thyroiditis with high titers (≧ 1 2500 by the indirect hemaggl [illegible] n method) of thyroglobulin auto-antibodies

2 Sera of patients without thyroid [illegible] r healthy pers ns with high titers (≧ 1 2500) of thyroglobulin auto ant b [illegible]

3 Sera from patients surgically tr a [illegible] thyroid disease from whom thyr [illegible] tissue was obtained

Hemagglutination The indirect hemagglutination procedure according t *B* [illegible] *den s* method (1) was employed Hum n erythrocytes were formaliniz [illegible] sensitized as previously descril ed (5 6 [illegible] The concentration of antigen u [illegible] r

sensitization was 0.5 mg per ml against 1.2 × 10^8 erythrocytes per ml. Such cells have been calculated to contain about 35000 antigen molecules per cell (7).

Hemagglutination inhibition. Inhibition of the hemagglutination was performed by addition of thyroglobulin or thyroid extracts immediately prior to addition of sensitized erythrocytes to serum dilutions in perspex trays. The reaction was read after incubation at 4° C over night.

Gel precipitation. The method of *Ouchterlony* (15) was performed as described by *Eriksen* (4) employing 0.03 M barbital buffer at pH 8.6 with 0.003 per cent methyl orange, 1 per cent Difco Special Agar Noble and 0.1 per cent phenol.

Estimation of protein. Protein concentration was determined by the biuret method as described by *Kabat & Mayer* (11).

RESULTS

Inhibition of Hemagglutination of Cells Sensitized with $(NH_4)_2SO_4$ Fractionated and Lyophilized Thyroglobulin by Fresh Extracts of Normal Thyroids

Milgrom & Witebsky (13) have shown that structural changes are produced in rabbit γ globulins by $(NH_4)_2SO_4$ fractionation. *Shulman & Armenia* (19) demonstrated that thyroglobulin is partially denatured by lyophilization. In the present study attempts were made to detect auto-antibodies to fractionated and lyophilized thyroglobulin not reacting with native thyroglobulin.

For this purpose 31 sera were tested against erythrocytes sensitized with fractionated and lyophilized thyroglobulin. Sixteen sera were obtained from individuals without evidence of thyroid disease, 14 from patients suffering from thyroid disease and the last serum was obtained from a patient (I. F.) who has previously been presented (8). This patient developed thyroglobulin antibodies to a titer of 1:2500 within a period of 6 months. As inhibiting antigen was used 0.01–0.02 ml per basin of a fresh thyroid extract from a normal thyroid containing approximately 40 mg protein per ml. This hemagglutination inhibition procedure was performed twice with thyroid extracts of different patients. On both occasions agglutination could be eliminated (< 1:25) from all sera tested. Similar extracts prepared from bovine thyroids did not interfere with agglutination.

Attempts to Demonstrate Antigenic Determinants Specific to the Thyroid of a Patient with Hashimoto's Thyroiditis

Six patients suffering from thyroiditis or myxoedema (J. B., S. A., A. M., B. A., L. B., I. S.) with a positive reaction by gel precipitation were tested against an extract of the thyroid of a histologically verified Hashimoto patient (E. I. G.) and an extract of a normal gland in adjacent wells. All sera gave rise to identity reactions between these 2 thyroid extracts as demonstrated by serum S. A. in Fig. 1.

Furthermore the same 31 sera used in the previous hemagglutination inhibition experiment were tested against erythrocytes coated with thyroglobulin prepared from the biopsy specimen of the Hashimoto

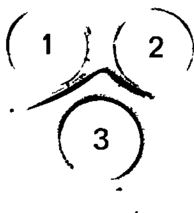


Fig 1

Precipitation in gel by serum S A (3) extract of a normal thyroid (1) and an extract of thyroid tissue from a case of Hashimoto s disease (2)

thyroid Agglutination of these cells which revealed the same sensitivity as cells treated with ordinary thyroglobulin could be eliminated (< 1 25) by a fresh extract of a normal gland

Inhibition of Agglutination of Erythrocytes Sensitized with Autologous Thyroglobulin by an Extract of a Normal Thyroid

From six patients with thyroid auto antibodies autologous thyroglobulin was prepared from thyroid tissue removed by surgical treatment Four of these patients suffered from thyrotoxicosis one from a simple colloid struma and the last one from Hashimoto s thyroiditis As shown in Table 1 cells coated with autologous thyroglobulin revealed the same sensitivity as cells coated with ordinary thyroglobulin and agglutination could be eliminated by an extract of a normal thyroid This extract had been kept at 4° C for 14 days before the experiment was performed

Cross Reactions of Human Auto ant[illegible] and Heterologous Thyroglobulins

Human thyroglobulin auto antibo[illegible] ave been shown not to cross react with heterologous thyroglob[illegible] by precipitation techniques (16) Using the method of indirect h[illegible]magglutination however cross reactions to a limited extent have [illegible] observed by *Rose & W[illegible]lebsk[illegible]* (18)

In the present study 2 out of 7 sera (S A and A M) from patients

with myxoedema or thyroiditis gave rise to extensive cross reactions as shown in Table 2. On the other hand serum H.A. and L.N. revealed no cross reacting antibodies. The other 4 sera showed limited degrees of cross reactions. This variability from one serum to the other is in agreement with the observations of *Rose & Witebsky* (18). The clinical significance of this variability at present remains unknown.

TABLE 1

Agglutination of Erythrocytes Coated with Autologous Thyroglobulin and Hemagglutination Inhibition by an Extract of a Normal Thyroid

Patient	Clinical diagnosis	Titer of agglutination		Hemagglutination inhibition	
		with autologous thyroglobulin	with homologous thyroglobulin	by human thyroid extract (0.01 ml/cup)	by bovine thyroid extract (0.01 ml/cup)
P.H.	Thyrotoxicosis	1 2500	1 2500	< 1 5	≥ 1 25
J.J.O.	"	1 25	1 25	< 1 5	≥ 1 25
J.M.	"	1 25	1 25	< 1 5	≥ 1 25
J.H.	"	1 5	1 5	< 1 5	≥ 1 5
G.S.	Non toxic goitre	1 25	1 25	< 1 5	≥ 1 25
I.I.G.	Hashimoto's thyroiditis	1 25	1 25	< 1 5	≥ 1 25

TABLE 2

Cross Reactions of Human Auto Antibodies with Heterologous Thyroglobulins

Patient	Clinical diagnosis	Hemagglutination titer of cells coated with thyroglobulin of				
		Man	Ox	Hog	Rabbit	Guinea pig
J.B.	Post irradiation myxoedema	1 25000		1 25	1 250	
S.A.	Thyroiditis	1 250000	1 2500	1 25000	1 25000	1 2500
A.M.	Thyroiditis	1 250000	1 50	1 2500	1 250	1 [illegible]
H.A.	Primary myxoedema	1 25000				
L.N.	Thyroiditis	1 25000				
I.B.	Thyroiditis	1 25000		1 250		
F.S.	Thyroiditis	1 2500000		1 250	1 250	

— < 1 25

Studies of rheumatoid factors by *Milgrom & Tonder* (12) have indicated that denaturation of autologous γ globulins may unmask hidden determinants which resemble antigenic determinants on the surface of heterologous γ globulins. In order to establish whether cross reacting antithyroglobulins also were directed against such determinants, a hemagglutination inhibition assay was performed as shown in Table 3. The 2 strongest cross reacting sera (S.A. and A.M.)

were tested in dilutions of 1:25 against heterologous and homologous thyroglobulins, the ability to inhibit these reactions by various types of thyroglobulins was studied. As homologous antigens were used both fractionated thyroglobulin from pooled diseased thyroids and an extract of a normal thyroid prepared 14 days before the present experiment.

TABLE 3
Hemagglutination Inhibition of Cells Coated with Various Kinds of Thyroglobulin by Homologous and Heterologous Thyroglobulin

Serum	Thyroglobulin used for inhibition of agglutination: Human	Extract of normal human thyroid	Bovine	Hog	Rabbit	Guinea pig	Cells coated with thyroglobulin from
A.M.	—*	—	+§	+	+	+	Man
tested	—	—	—	+	—	+	Ox
in a	—	—	+	—	+	+	Hog
dilution	—	—	+	+	—	+	Rabbit
of 1:25	—	—	+	+	—	—	Guinea pig
S.A.	—	—	+	+	+	+	Man
tested	—	—	—	+	—	+	Ox
in a	—	—	+	—	+	+	Hog
dilution	—	—	+	+	—	+	Rabbit
of 1:25	—	—	+	+	—	—	Guinea pig

* — = agglutination inhibited
§ + = agglutination not inhibited

The hemagglutination inhibition studies show that all the cross reacting antibodies may be removed by absorption with homologous thyroglobulins. In contrast heterologous thyroglobulins in general only inhibit cells coated with the same type of thyroglobulin. An exception is made by rabbit thyroglobulin which in addition inhibited cells coated with bovine and guinea pig thyroglobulin.

Of the other sera only the cross reactions between J.B. and F.S. and rabbit thyroglobulin were studied by hemagglutination inhibition. These reactions likewise were inhibited by the extract of the normal thyroid.

DISCUSSION

The present studies have failed to detect populations of antibodies against autologous thyroglobulin, homologous $(NH_4)_2SO_4$ fractionated and lyophilized thyroglobulin, thyroglobulin from a Hashimoto diseased thyroid or heterologous thyroglobulin which do not react with an extract of a normal thyroid.

One explanation of the negative findings could be that even a gentle preparation of a thyroid extract may unmask antigenic determinants against which auto antibodies are directed. Since components

diseased autologous thyroids or the thyroid of a patient with Hashimoto's disease could be detected

Sera from patients with thyroiditis or myxoedema gave rise to a variable degree of cross-reactions with heterologous thyroglobulins Hog and rabbit thyroglobulin coated erythrocytes were found to be agglutinated more frequently and gave rise to higher titers than cells coated with guinea pig and bovine thyroglobulin

The method of hemagglutination inhibition indicated that the cross reacting antibodies represented separate populations of antibodies It is estimated that human auto-antibodies may at least react with 5 antigenic determinants of different specificity, all of which are present on "native" thyroglobulin molecules

The present studies have failed to demonstrate human auto anti bodies reacting with individual specific determinants or determinants specific to structurally altered thyroglobulin molecules

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Acta path et microbiol scandinav 69, 214-218, 1967

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EVIDENCE FOR NON-RIBOSOMAL LOCATION OF STREPTOMYCIN RESISTANCE IN *ESCHERICHIA COLI* CARRYING THE MU-FACTOR

By

WENCHE BLIX GUNDERSEN

Received 14 vii 66

The presence of the Mu-factor in cells of *Escherichia coli* furnishes these cells with two possible levels of protection against the lethal effect of streptomycin When the Mu-factor is carried in its extra-chromosomal state (11) the cells have a low level resistance to streptomycin (9), the actual concentration of streptomycin tolerated depends upon the physiological state of the cells In 10^{-4} of the cells in such a population the chromosome is modified, possibly by an attachment of the Mu factor to the bacterial chromosome, resulting in a significantly higher level of streptomycin resistance (11, 8). Experiments on uptake of ^{14}C-streptomycin (10) indicate that the low-level streptomycin resistance may be due to a decreased permeability of the drug The higher level of resistance, however, could not be explained exclusively by a lack of penetration The uptake of streptomycin found in these cells seemed higher, rather than lower, than in the low-level resistant cells, suggesting the possibility of an additional mechanism of streptomycin resistance in these cells

The mechanism of true, one-step streptomycin resistance has been shown by several investigators to be due to an alteration in the structure of the ribosomes, rendering them insensitive to the interfering action of streptomycin (4, 5, 6, 1, 2) *Rosenkranz* (16) has shown that ribosomes from cells of *E coli* carrying the "multiple drug resistance" factor are inhibited by streptomycin to the same extent as ribosomes from ordinary streptomycin sensitive cells suggesting another mechanism of streptomycin resistance in these cells The author proposes a lack of penetration

If the low level streptomycin resistance in the cells carrying the Mu-factor in its extrachromosomal state is solely due to a lack of penetration one should expect to find their ribosomes as sensitive to streptomycin as are ordinary, sensitive ribosomes The high level resistance, however seems to depend upon an additional mechanism of protection Could this mechanism be an alteration of the ribosomes? The present study was carried out in order to elucidate this question

MATERIALS AND METHODS

Bacterial strains K12 T71 T71 str r T71Mu T71Mu str r and T71Mu thr his str r have formerly been described (11 8) The bacteria were grown in Heart Infusion Broth (Difco)

Preparation of cell extracts Cell extracts (S-30) were prepared essentially as described by *Nirenberg & Matthaei* (13) The washed cells suspended in twice their wet weight of standard buffer (13) were disrupted by treatment at 1 5 amp twice for 50 seconds in an M S E ultrasonic disintegrator (60 watts) (20) Before this treatment the cell suspension was cooled to 0° C and during the treatment it was

[illegible]

peptide The
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ate 6 μmoles
10 mμmoles

(0 5 μc) Controls lacking poly uridylic acid [illegible] were always carried out simultaneously All values were corrected for a non incubated control reaction terminated at zero time The reaction was run at 37° C Streptomycin when used was added at zero time poly U was added 20 minutes later The reaction was terminated at 45 min by the addition of two volumes of 10 per cent trichloracetic acid containing 1 mg of l phenylalanine per ml The tubes were then heated for 10 min at 95° C cooled filtered and washed with 5 per cent trichloracetic acid on Mill pore filters (0 45 mμ pore size) mounted on aluminium planchets and counted on a Frieseke and Hoepfner windowless gas flow counter

Biochemical and radioactive materials Disodium adenosine triphosphate (ATP) and l phenylalanine were purchased from Sigma Chemical Co St Louis Mo Trisodium guanosine triphosphate (GTP) disodium creatine phosphate creatine phosphokinase (ex skeletal rabbit muscle) and 2 mercaptoethanol were products of Koch Light Laboratories Colnbrook Bucks England Poly uridylic acid was a gift from dr L O Froholm State Institute of Public Health Oslo Norway Streptomycin sulphate was produced by Glaxo Laboratories Ltd. Greenford England The 1 3 phenylalanine C^{14} (uniformly labelled) was purchased from The Radiochemical Centre Amersham Bucks England

RESULTS AND DISCUSSION

It was not surprising to find that cell extracts from cells of E coli carrying the Mu factor in its extrachromosomal state (11) were inhibited in the poly U stimulated synthesis of polyphenylalanine by streptomycin to the same extent as ordinary streptomycin sensitive cells (Table 1) The low level streptomycin resistance shown by the cells in this condition has already been accounted for as an impaired streptomycin uptake (10) The mechanism of the low level streptomycin resistance furnished by the Mu factor in its extrachromosomal state may be identical to the Sm resistance carried by the multiple drug resistance factor (23 16)

In the state where the Mu factor modifies the bacterial chromosome possibly by an attachment to it (8) the cells achieve resistance to around 250 μg streptomycin per ml when growing in complete medium The results of the present investigation indicate that ribosomes from these cells are unchanged as compared to streptomycin sensitive ones they are inhibited by streptomycin in their poly U stimulated synthesis of polypeptides as are ribosomes from sensitive cells There are several conceivable ways to explain these findings

TABLE 1

Effect of Streptomycin on Poly U-Stimulated Synthesis of Polyphenylalanine by Cell-Free Extracts from E. coli Carrying the Mu factor

Extract	Strep concentration x 10^{-6} M	L-phenylalanine inc c p m	Inhibition per cent
T71Mu	0	2787	
	2	1809	36
T71Mu str-r	0	1067	
	2	695	35
T71Mu thr his str-r	0	1090	
	2	669	38

The experimental conditions are described in "Materials and methods". The content of C^{14} phenylalanine per sample gave 115 000 c p m The aliquots of S 30 fractions contained *T71Mu*, 2 1 mg, *T71Mu str-r*, 2 0 mg and *T71Mu thr his str r* 1 7 mg bacterial protein per sample

The ordinary streptomycin sensitive strain *E coli K12 T71* was inhibited to the same extent by this concentration of streptomycin (cfr 16) whereas the ordinary resistant mutant, *K12 T71 str-r* was completely unaffected

Whereas both the mutator-streptomycin resistant mutants used in the present study, as well as all the ones tested, tolerate around 250 µg streptomycin per ml, the ordinary one-step streptomycin resistant mutant used, *K12 T71 str-r*, has been found to grow unimpaired in the presence of 5000 µg streptomycin per ml, and it also grows, although somewhat more sparsely, in the presence of 20 000 µg streptomycin per ml The S-30 fraction from this mutant is completely unaffected in the *in vitro* polyphenylalanine synthesis by 2×10^{-6} M streptomycin This is in accordance with results from other laboratories (10, 18, 16, 1 22) Only in one case (22) the actual level of resistance of the streptomycin resistant strain used has been reported, and in that case the tolerance of the strain was 3–5 mg streptomycin per ml It is, however, a generally accepted fact that the true, one-step streptomycin resistant strains have a high level resistance (12, 17) Thus, one possible explanation is that a modification of the ribosomes of the mutator-streptomycin resistant cells, increasing the level of resistance from 15 to 250 µg streptomycin per ml results in a difference too small to be registered by the present experimental system

It seems beyond doubt that streptomycin really interferes with the action of the ribosomes, causing the formation of aberrant protein, the evidence supporting this hypothesis is overwhelming (6, 7, 2, 14) These findings are also consistent with the genetic facts of the streptomycin system, the streptomycin sensitivity, resistance and dependence being three alleles of the same gene This situation has been very hard to reconcile with some of the other theories on streptomycin action There is, however, some evidence indicating that the formation of aberrant protein may not be the sole lethal action of streptomycin (21)

there may be an additional more general reaction of of streptomycin with the nucleic acids (15)

Genetic evidence has been presented (8) indicating that the mutator streptomycin resistance is located at the bacterial chromosome at a site distinct from that of the ordinary streptomycin locus and apparently there is also a certain genetic distance between the two loci The mutant *T71Mu thr his str r* has been employed in the present study because of its former use in the genetic mapping experiments The genetic facts agree with two possible explanations of the present results The normal gene for streptomycin sensitivity is present in the mutator streptomycin resistant cells (8), a fact that might imply that their ribosomes are unchanged The possibility of this resistance being due to a suppressor mechanism has formerly been suggested (8) This would result in a quantitative rather than a qualitative change which is reconcilable with the present experimental data However, the present results do not exclude the existence of a third different mechanism of streptomycin resistance in addition to the well established ribosomal resistance and the mechanism of decreased permeability

SUMMARY

The action *in vitro* of streptomycin on poly U stimulated polypeptide synthesis has been studied with cell free extracts from Escherichia coli carrying the Mu factor in the extrachromosomal as well as in the possibly attached state The polypeptide synthesis was inhibited by streptomycin like that found in cell free extracts from streptomycin sensitive organisms suggesting another mechanism of resistance than the ribosomal resistance of the true one step streptomycin resistant mutants

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Acta path et microbiol scandinav 69 219 236 1967

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STUDIES ON THE EPIDEMIOLOGY OF STAPHYLOCOCCAL INFECTIONS

3 *Influence of Factors Inherent in the Patient and his Operation*

By

GUNNAR LINDBOM GUNNAR LAURELL and ÅKE GRENVIK

Received 22 vii 66

In the Department of Thoracic Surgery, the epidemiology of nosocomial staphylococcal infections has been investigated since 1958 The study covers a period when—due to rapid progress in surgery and related fields—the composition of the relevant population has materially changed Extracorporeal circulation has enabled surgical correction of many cardiac lesions, both congenital and acquired, which were formerly regarded as inamenable This has resulted in an increase in the number of lengthy, radical and technically complicated operations on the heart Concurrently, a decrease in surgery on patients with pulmonary tuberculosis has taken place Moreover the indications for cardiac operations have widened The willingness of surgeons to operate on patients who were recently regarded as too old or too debilitated has, in fact led to a steady rise in the proportion of high-risk patients Finally as a result of cumulative experience, stricter standards of hygiene have been imposed, and various preventive measures against sepsis have been tested

... belonged to the Ps. 52A/80/81 complex were characterized by the 75/77 pattern or were non typable (*Lindbom* 1964) If allowance is made for changes in surveillance during the period little evidence suggests that the total incidence of sepsis had been influenced by the increasing number of extensive operations in high risk patients It was nevertheless the clinical ...

... published surgical experience contamination of the wound during operation was infrequent In 1961 approximately 2/3 of all patients were found to have acquired the infecting strains outside the operating theatre About 1/3 of the septic cases were classified as autoinfection Human sources healthy or infected played a critical rôle in the intensive-care ward during the early postoperative phase and frequently initiated a vicious circle of cross infection

PLAN OF THE PRESENT STUDY

Although calculation of the over all rate of postoperative infections reflects the total picture of sepsis it fails to identify particularly infection prone groups of patients The method of surveillance therefore included relevant clinical and bacteriological data, to permit calculations of specific infection attack rates It was drawn from three sources wound healing cards laboratory notes, and case records

Most patients passed through several hospital environments in the unit *i e* the nursing ward, the operating theatre, and the intensive care ward, where they were exposed to a variety of conditions for varying periods Previous studies had emphasized the importance of the early postoperative phase in the intensive care ward Hence the interaction was to be studied between postoperative patients and the environmental pool of staphylococci as well as the dependence of the environmental flora on the presence of staphylococcal disseminators

In view of the high rate of autoinfection in 1961, special attention was also focused on the nasal carrier state of the patients, and nasal chemotherapy was instituted

The results and conclusions of the study will be presented in three reports dealing with different aspects (a) the influence of factors inherent in the patient and his operation on the rate of postoperative sepsis (b) the influence of nasal chemotherapy on the carrier state in patients and (c) the influence of environmental factors during the early postoperative phase

MATERIAL AND METHODS

The lay-out of the wards and the basic bacteriological technique were the same as those previously described (*Lindbom* 1964) but the epidemiological survey was more comprehensive It included regular carrier examinations, standardized environmental examinations in the intensive care ward and a pilot study of the *operating theatre*

Background

General Observations The study took place from January 1st to December 31st 1962 inclusive A total of 536 patients were admitted to the unit There were 313 operations Their duration ranged from 1 2 9 6 hours (average 3 3) Due to the high incidence of lengthy procedures on the open heart the average number of operations per operating day usually ranged from 1 1 1 3 In May June, September and December it rose to 1 6–1 7 because of an increased number of relatively short operations

The "average" patient remained in the unit for 29 5 days, of this time he spent 9 5 days in the ward undergoing a preoperative work up 4 5 days postoperatively in the intensive-care ward and, finally another 15 5 days in the ward before being transferred to another unit or another hospital Table 1 shows the average duration of these three phases for certain cardiac and pulmonary operations

Respirator Treatment A total of 80 patients were tracheotomized The duration of treatment ranged from 1 51 days, in 2/3 of the patients it lasted for 3 8 days (average 7 8)

Antimicrobial Policy Prophylactic antibiotics were given routinely to about 50 per cent of the patients preoperatively and to over 90 per cent postoperatively Penicillin G and chloramphenicol were the drugs of choice and were frequently combined Penicillin was given to 20 per cent of the patients preoperatively and to 32 per cent pos [illegible] per cent and 41 per cent respectively Othe [illegible] secondary importance They were generally [illegible] chloramphenicol

In open hear [illegible] alve had been replaced by a Starr Edwards prosthesis the patient received penicillin and chloramphenicol for a week after operation via a catheter in the superior vena cava

Nasal Disinfection [illegible] chlorhexidine neomycin c [illegible]

Collection of Data [illegible] pendent sources *i e* wound l [illegible] lococci isolated in the unit [illegible] of the

case records of all operated patients They were to serve as a control of the efficiency of the other two sources, and to permit calculations of specific infection attack rates

Selection of Patients All patients whose postoperative course was complicated by infection were included The epidemiological study dealt only with patients who acquired staphylococcal infections

TABLE 1

Average Duration of Phases of Hospitalization in Some Common Cardiac and Pulmonary Operations

Surgical procedure	Phases of hospitalization		
	Preoperative days	Operative hrs	Postoperative days
Total valve prosthesis	12	5.2	20 (11)
Transventr dilatation	10	2 3	17 (4)
Closure of ASD	5	2 6	12 (4)
Closure of VSD	10	3 9	25 (5)
Beck's operation	25	1 7	23 (4)
Resection of aortic coarct	4	2 6	6 (3)
Division of patent ductus	0	2 1	6 (3)
Pulmonectomy	15	3 5	20 (6)
Lobectomy	14	3 3	12 (3)
Segmental resection	13	2 9	13 (5)
Expl thoracotomy	10	2 2	14 (4)

Bracketed figures denote duration of postoperative intensive care

The final decision whether a patient should be classified as infected was made by a thoracic surgeon (G k), who saw most patients and participated in their operations In general, the classification presented no problem The absence of a bacteriological verification would however have excluded several suspect cases of septicaemia where clinical data and/or microscopical diagnosis were accepted as presumptive evidence of septicaemia

Definitions A postoperative *wound infection* was defined as a complication in which bacteria were not only present in the incision, but also elicited a local or systemic [illegible]
systemic [illegible]
have bee [illegible]
When [illegible]
leucocytosis—was present in a patient more than 5 days after operation he was classified as a presumptive case In patients receiving antimicrobials directly via vena cava catheters bacteriological verification was frequently impossible The aetiological diagnosis was inferred from the presence of staphylococci elsewhere in concomitant septic lesions such as empyema tracheal secretion, and a septic wound

Partial atelectasis was frequent postoperatively Varying degrees of pulmonary opacification on chest X rays and "infected sputum were not accepted as reliable criteria of bronchopneumonia Consequently only cases where the diagnosis was established at autopsy are included

The term *staphylococcal diarrhoea* denotes a postoperative condition characterized by moderate to profuse stools from which enterotoxin B producing staphylococci were isolated

Staphylococcal disease was classified as *minor* when due to a trivial wound infection whose main importance was epidemiological, *i e*, as a potential source of pathogens *Major* infections were associated with septicaemia, empyema, sternal osteitis, staphylococcal diarrhoea or wounds with invasion and destruction of tissue

Bacteriological Routine

Carrier Examination (a) Patients —Nasal and throat swabs were taken from patients on the day of admission or as soon after as possible Patients were also

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In open heart surgery when a diseased cardiac valve had been replaced by a Starr Edwards prosthesis the patient received penicillin and chloramphenicol for a week after operation via a catheter in the superior vena cava

Nasal Disinfection All patients underwent nasal chemotherapy using a chlorhexidine neomycin cream and/or a framycetin gramicidin nasal spray

Collection of Data In 1961 information was collected from two independent sources *i e* wound healing cards and continuous registration of all staphylococci isolated in the unit In the present study a third source was added a review of the

diagnosis in 3 cases where post-mortem findings supported the diagnosis of septicaemia The diagnosis of "bronchopneumonia" in 6 cases was based solely on the post-mortem examination, bacteriological verification was possible in 3 cases, whereas no specimens were obtained from the remaining 3

TABLE 2

Total Spectrum of Infectious Complications in 58 Patients

Type of complication	Clinical diagnosis Verified	Not verified	Total no
Wound	39	5	44 (18)
Wound abscess	3	3	6 (3)
Retrosternal abscess	2	1	3
Osteitis	1	1	2
Bronchopneumonia	3	3	6 (2)
Empyema	4	–	4 (3)
Septicaemia	7	10	17 (6)
Staph. diarrhoea	6	–	6 (2)
Total	65	23	88 (34)

Bracketed figures denote number of single lesions

TABLE 3

Postoperative Infections Grouped According to Site of Operation

Site of operation	Infections Major	Minor	No of infections
Heart and great vessels	23 (15)	17	40
Lungs	5 (2)	4	9
Miscellaneous	7 (2)	2	9
Total no of infections	35 (19)	23 (1)	58

Bracketed figures denotes number of deaths

Importance of Postoperative Infection Septic complications varied from minor episodes in 23 patients to major illness in 35 patients, which contributed to the death of 19 Two-thirds of all infections were classified as "major" (Table 3)

In 23 patients who were seriously ill with staphylococcal disease, the course was progressive, with a tendency to necrosis, abscess formation and septicaemia It could be arrested in 7 patients by intense antimicrobial therapy or additional operations but progressed rapidly to death in the remainder Tissue destruction subsequent to sepsis nullified the expected result of surgery in 6 patients, and contributed to th death of 5

Only a minority of patients remained in the unit for more than a

swabbed on the day of operation and daily during their stay in the intensive-care ward (b) Personnel — Nasopharyngeal swabs were obtained from regular members of the personnel every other month

Septic Complications As soon as clinical symptoms and signs suggestive of infection appeared, specimens were collected for culture, to verify the diagnosis and to determine the aetiological agent

Bacteriological Technique The bacteriological technique was described in earlier reports (*Laurell & Lindbom* 1961) The term "staphylococci" as used in this report refers only to coagulase positive strains All staphylococcal were phage typed with the basic set of 21 international phages in addition to phage KS6 (*Wallmark* 1954) The technique was that described by *Blair & Williams* (1961). The disc diffusion method of *Ericsson et al* (1954) was used for sensitivity determinations

Formation of enterotoxin B was determined by a method described by *Hallander* (1965) Staphylococci to be examined were streaked onto solid media covered with cellophane, and incubated for 24 hours The supernatants obtained after centrifugation were tested for the presence of enterotoxin by the gel diffusion technique

Bacteriological Data Information in this study derived from approximately 8000 cultures including 1179 clinical specimens and 6716 specimens collected to map the epidemiological background

Part 1 of the study, which deals with the patient and his operation is based on 210 strains of staphylococci isolated from clinical specimens

RESULTS

1 *Infectious Complications*

Incidence of Infection Postoperative infection developed in total in 58 among 313 operated patients *Staph aureus* was isolated from 41 The over all infection rate was 18 5 per cent, the staphylococcal infection rate 13 1 per cent The total number of septic lesions in these 58 patients was 88 Single lesions occurred in 34 patients and multiple in 24

Efficacy of Surveillance Methods Only by a thorough review of case records could 58 cases of postoperative septic complications be disclosed If this is accepted as the true number of infections, the accuracy of "wound-healing cards" was just over 65 per cent (38 cases), and that of bacteriological records about 74 per cent (43 cases) As information from these two sources did not completely overlap, they detected just under 80 per cent (46 cases) of all infections The inadequacy of the reporting system was due either to the absence of cards concerning emergency operations, operations on children and those not strictly on the heart and lungs, or to inadequate or missing cultures from suspected septic lesions

Reliability of Classification The extent to which a clinical diagnosis could be confirmed bacteriologically is shown in Table 2 Verification was possible in about 74 per cent of all lesions classified as septic Whereas the diagnosis was confirmed in 89 per cent of all septic wounds, that of septicaemia had to be based on clinical data in 10 of 17 cases All unconfirmed cases had received massive doses of antibiotics (including methicillin) when blood samples were collected The outcome was fatal in 6 verified and 8 unverified cases Bacteriological cultures from relevant autopsy tissues failed to confirm the clinical

diagnosis in 3 cases where post-mortem findings supported the diagnosis of septicaemia The diagnosis of "bronchopneumonia" in 6 cases was based solely on the post mortem examination, bacteriological verification was possible in 3 cases whereas no specimens were obtained from the remaining 3

TABLE 2
Total Spectrum of Infectious Complications in 58 Patients

Type of complication	Clinical diagnosis		
	Verified	Not verified	Total no
Wound	39	5	44 (18)
Wound abscess	3	3	6 (3)
Retrosternal abscess	2	1	3
Osteitis	1	1	2
Bronchopneumonia	3	3	6 (2)
Empyema	4		4 (3)
Septicaemia	7	10	17 (6)
Staph diarrhoea	6	–	6 (2)
Total	65	23	88 (34)

Bracketed figures denote number of single lesions

TABLE 3
Postoperative Infections Grouped According to Site of Operation

Site of operation	Infections		No of infections
	Major	Minor	
Heart and great vessels	23 (15)	17	40
Lungs	5 (2)	4	9
Miscellaneous	7 (2)	2	9
Total no of infections	35 (19)	23 (1)	58

Bracketed figures denotes number of deaths

Importance of Postoperative Infection Septic complications varied from minor episodes in 23 patients to major illness in 35 patients, which contributed to the death of 19 Two thirds of all infections were classified as "major (Table 3)

In 23 patients who were seriously ill with staphylococcal disease, the course was progressive with a tendency to necrosis, abscess formation and septicaemia It could be arrested in 7 patients by intense antimicrobial therapy or additional operations but progressed rapidly to death in the remainder Tissue destruction subsequent to sepsis nullified the expected result of surgery in 6 patients, and contributed to the death of 5

Only a minority of patients remained in the unit for more than a

few weeks after operation, although several with serious infections had to be hospitalized for periods ranging from 2–4 months

Staphylococcal Diarrhoea Of particular interest were 6 patients with postoperative diarrhoea In 4, it was part of a clinical picture that included a septic wound combined with bronchopneumonia or septicaemia All patients had positive cultures of *Staph aureus* in specimens of their stools, and concomitant septic lesions The strains isolated were identified by phage typing as 75 77 or Thorax I (*cf* the following) Both were found to produce enterotoxin B An interesting feature was the frequent association of diarrhoea with tracheobronchitis and/or bronchopneumonia Thus, severe staphylococcal disease characterized by simultaneous infection of the gastro intestinal and respiratory tract occurred in 4 patients, 3 of whom later succumbed to their infection It was typical of this disease pattern that patients harboured and liberated large numbers of staphylococci and caused massive environmental contamination Further details of the epidemiological importance of these *disseminators* will be published separately (*Lindbom & Laurell*)

It is noteworthy that although enterotoxic strains produced septic complications in a total of 16 patients (including 2 double infections), there were only 2 cases of isolated staphylococcal diarrhoea In the others, the clinical picture was a combination of infection of mucous membranes, usually associated with septicaemia and other severe forms of disease

Association Between Type of Operation and Rate of Sepsis

Heart and Great Vessels In total 175 operations were performed Certain operative characteristics were used to divide the material into two main groups open and closed heart operations (Table 4) These were further divided into two subgroups, *i e* acquired and congenital defects, in which operations were listed according to the underlying disease

Whereas operative correction of congenital cardiac deformities is fairly well standardized it is in a developmental stage for acquired lesions For technical information on cardiac operations reference is made to *Björk* (1964) It should be noted that valvular replacement was performed only in advanced cases Respirator treatment was used in patients with an enlarged heart and pulmonary hypertension to lessen respiratory work and diminish the cardiac output Seventy patients were tracheotomized when prolonged treatment was anticipated

The term open heart operation covers various intracardiac procedures performed on 95 patients Extracorporeal circulation (ECC) was used in 76 and hypothermia in 26 in 7 hypothermic patients ECC was subsequently found to be necessary Altogether 43 patients had acquired mitral and/or aortic valvular deformities Prosthetic replacement was performed in 41 patients mitral annuloplasty in 1 and aortic commissurotomy in 1 A Starr Edwards ball valve prosthesis was used in 38 operations including 2 re operations to replace 32 aortic and 10 mitral valves A double aortic and mitral prosthesis was used in 2 patients A Muller prosthesis was used in 2 patients and a Hufnagel prosthesis in 1 Hypothermia was used

in 20 patients with an atrial septal defect (with or without anomalous pulmonary return), 3 with pulmonary stenosis and 3 with coarctation of the aorta.

TABLE 4

Operations on the Heart and Great Vessels

Operative procedure	Structural changes	No. of Operated cases	No. of Infected cases	Infections %
Open heart operation	*Acquired*			48.9
	AI ± AS	29	14	
	MI ± MS	10	3	
	AI ± MI	4	4	
	Ventr. aneurysm	3	1	
	Misc.	1	1	
	Congenital			22.9
	Tetralogy of Fallot	4	1	
	VSD	13	3	
	ASD (sec.)	23 (20)	5 (4)	
	Misc.	8 (3)	2	
Closed operation	*Acquired*			6.6
	MS	12	0	
	Angina pectoris	11	2	
	Adams Stokes syndrome	29	1	
	Misc.	9	1	
	Congenital			10.5
	Coarctation of aorta	8	2	
	Patent ductus art.	11	0	
Total		175	40	22.9

A aortic
M mitral
I insufficiency
S stenosis
ASD atrial septal defect
VSD ventricular septal effect

Bracketed figures denote number of operations under hypothermia.

The over all rate of sepsis in this group of 175 patients was 22.9 per cent. It was 35.8 per cent in patients undergoing open heart surgery, 48.9 per cent in patients with acquired valvular deformities as compared to 22.9 per cent in those with various congenital anomalies. Postoperative infection occurred in 21 patients (53.5 per cent) with a ball valve prosthesis: in 17 cases after replacement of aortic valves and in 5 after mitral replacement. Sepsis developed in only 4 of 26 hypothermic operations (15.4 per cent).

It is noteworthy that 27 of 70 tracheotomized patients (38.6 per cent) became infected.

Closed cardiac operations carried an infection rate of 7.5 per cent. Four patients with acquired lesions and 2 with congenital deformities

became infected. Two patients out of 11 undergoing Beck's operation developed sepsis. Wound sepsis occurred in one patient, and staphylococcal diarrhoea in the other, whose parietal wound was perfectly healed. Resection of aortic coarctation in 8 patients was followed by trivial sepsis in 2. In contrast, 11 patients with a patent ductus and 12 with mitral stenosis remained free from postoperative sepsis.

Operations associated with a high rate of sepsis were usually of long duration. On an average, open correction of acquired valvular deformities, ventricular (VSD) and atrial septal defect (ASD) lasted for 5.2, 3.9 and 2.6 hours, respectively. There was, however, no direct evidence of a time-risk relation, since ASD and VSD operations were associated with a similar rate of sepsis, despite a marked difference in duration, whereas transventricular dilatation of stenotic mitral valves (averaging 2.3 hours) remained entirely free from septic complications. Furthermore, the use of extracorporeal circulation did not in itself predispose to sepsis, as congenital defects made accessible to surgery by hypothermia (ASD) and E.C.C. (VSD) were accompanied by similar attack rates, 20 per cent and 23 per cent, respectively.

A closer correlation seemed to exist between the rate of sepsis and patient factors. Thus, various host characteristics, such as duration of underlying disease and cardiac reserve, showed a positive correlation to the incidence of sepsis. Valvular replacement by a prosthesis was performed only in advanced cases of enlarged heart (700-900 ml/m., BSA) in patients who frequently had pulmonary hypertension and some degree of myocardial failure. Generally, they were in need of prophylactic respirator treatment and were tracheotomized in the theatre.

Clinically, infections were trivial in 5 patients and severe in 16 after prosthetic replacement of diseased valves. They contributed to the death of 13 patients. Generally, they were due to a combination of a septic wound and/or a deep abscess with septicaemia. Most clinical specimens yielded a polybacterial flora of *Staph aureus*, *Staph albus* and/or enteric gram-negative bacteria. The last-mentioned were isolated only from patients undergoing therapeutic methicillin treatment. In contrast, only 2 septic complications subsequent to correction of congenital cardiac anomalies were classified as serious. Sternal osteitis developed in one patient, and a retrosternal abscess in the other. Cultures yielded *Staph aureus* alone or in combination with *Staph albus*.

More than 45 per cent of the septic complications following prosthetic replacement of deformed valves were due to autoinfection.

Closed operations were associated with 5 minor wound infections and 1 case of isolated staphylococcal enterocolitis.

Lungs. Data on 72 pulmonary operations are summarized in Table 5. The main indications for surgery were malignant tumours (25 cases), tuberculosis (20 cases), and bronchiectasis (6 cases). Pulmonectomy

TABLE 5

Pulmonary Operations

Clinical diagnosis	Pulmonectomy ± Thoracoplasty		Lobectomy ± Segment resection ± Thoracoplasty		Segmental resection		Other operations incl thoracotomy		Infections %
	Operated cases	Infected cases	Operated cases	Infected cases	Operated cases	Infected cases	Operated cases	Infected cases	
Tuberculosis of lung	2	0	14	1	3	0	1	0	5.0
Tumour of lung	20	6	4	0	0	0	8	1	21.9
Bronchiectasis	0	0	6	1	0	0	0	0	16.7
Other pulm processes	0	0	0	0	2	0	12	0	0
Infections %	27.3		8.3		0		4.8		12.5

was performed in 21 cases and lobectomy in 22 About 90 per cent of the former were performed on patients with malignant tumours and 60 per cent of the latter on patients with tuberculosis

Both pulmonectomy and lobectomy involved transection of previously infected tissues Atypical infections distal to cancerous bronchial obstruction were common prior to surgery in pulmonectomized patients The average age was also higher (46 2 years) than that in lobectomized patients (35 7 years) As respiratory difficulties were anticipated in 7 patients they were tracheotomized to permit the use of a mechanical ventilator Intercostal drainage tubes were placed in the thorax to maintain pulmonary expansion

The total number of postoperative infections was 9 giving a total sepsis rate of 12 5 per cent Postsurgical infections complicated 27 3 per cent of all pulmonectomies and 8 3 per cent lobectomies

Infection attack rates for pulmonectomy and lobectomy were not directly related to the length of operation (3 5 and 3 3 hours respectively) Again factors inherent in the patient—such as age underlying disease previous infection of the lung nature and extent of ventilatory disturbances—tended to obscure the influence of increasing duration of operation

After pulmonectomy infectious complications appeared in 6 patients with malignant tumours They were serious in 5 and a contributory cause of death in 2 All patients had been tracheotomized The clinical picture was characterized by septic wounds plus tracheobronchitis bronchopneumonia and in some cases empyema Clinical specimens yielded both *Staph aureus* and gram negative enteric bacteria A trivial wound in the 6th patient—who had not been tracheotomized was due to *E coli* and *Staph albus*

Minor septic wound from which *Staph albus* and gram negative bacteria were isolated developed in 2 lobectomized patients

Miscellaneous In this heterogeneous group of patients 9 out of 66 operations were complicated by postoperative infection 5 were associated with vascular operation 2 with tracheal and 1 with gastrointestinal The total incidence of sepsis was 13 6 per cent

The clinical picture was usually characterized by a septic wound or an abscess There was only 1 case of septicaemia Surgery on the aorta and trachea was complicated by 5 serious infections 2 of which ended fatally *Staph aureus* was the dominant organism Two thirds of all infections were monobacterial ⅓ due to mixtures of *Staph aureus* *Staph albus* and less commonly to enteric gram negative organisms

Tracheostomy Septic complications developed in 34 out of 80 tracheotomized patients (42 5 per cent) 23 were caused by *Staph aureus*

2 *Bacteriological Findings*

The results of bacteriological examination of clinical specimens are summarized in Table 6 Pure cultures were found in 33 patients with postoperative infections and mixed cultures in 25 *Staph aureus* was the predominating organism It was isolated from 70 7 per cent of all infections while *Staph albus* was found in 36 3 per cent and various enteric gram negative bacteria such as *E coli* *Proteus* and *Pseudomonas* in 25 9 per cent *Staph aureus* was thus about twice as frequent as *Staph albus* and three times as frequent as gram negative bacteria Their relative aetiological importance *i e* their ability to initiate infection under existing conditions was estimated in 33 patients with monobacterial sepsis *Staph aureus* had produced 75 6 per cent of these infections causing more than 3 times as many infections as *Staph albus* and 25 times as many as enteric gram negative bacteria

TABLE 6

Bacteria Isolated in Postoperative Infectious Complications in 55 Patients *

Organisms	No of infectious complications
Staph aureus	41 (25)
Staph albus	21 (7)
Gram negative bacteria	15 (1)
None	1
	78 (33)

No material for bacteriological examination was obtained from 3 patients

Bracketed figures denote number of pure cultures

Phage Typing Results By means of phage typing 81 different lytic patterns could be distinguished by conventional principles of strain differentiation About 60 per cent of all staphylococci had to be retyped at RTD × 1000 resulting in a great number of somewhat broad lytic patterns that were often difficult to interpret The main problem was presented by two strains characterized by wide pattern involving phages of groups I and III One has [illegible] denoted as Thorax "BP (basic pattern) Its patterns (including moderate reactions) were basically alike RTD × 1000 52 52A 80 KS6 7 42E 53 54/75 77 83A but did not show complete correspond[illegible] D[illegible]viations from this basic pattern involving the gain or loss [illegible] [illegible]trong reactions occurred frequently in a set of cultures obtain[illegible] [illegible]t intervals from the same patient Strains with this pattern hav[illegible] [illegible]een regarded as related without further epidemiological eviden[illegible] D[illegible]stinct from the "BP" staphylococcus was a strain Thorax "I[illegible] [illegible]ibition) which was characterized by reactions of inhibition produced by phages 81 KS6 6 47 83A and less commonly 52 52A 7 42E and 75 Although deviations from the

pattern of inhibition reactions were common, these strains showed fairly good reproducibility

Among 81 recognizable staphylococcal strains, 24 were recovered from various septic lesions in 41 patients Five distinct strains produced more than 2 infections each They have been denoted as "epidemic" Such strains, recovered from 15 septic cases, were identified by well-defined lytic patterns, *i e.* the 52/52A/80 81 complex (4 cases, 9 8 per cent), 75/77 (8 cases, 19 5 per cent) and RTD × 1000: 53 42E (3 cases, 7 3 per cent) Staphylococci characterized by wide complex patterns were recovered from 18 patients, Thorax BP (8 cases, 19 5 per cent) and Thorax I (10 cases, 21 4 per cent) As cultures from 3 patients with 75/77 infections also yielded either Thorax BP or Thorax I strains, these 5 epidemic strains together accounted for 31 infections, *i e*, 75 6 per cent of all staphylococcal infections The remaining 10 cases were all sporadic infections

Antibiograms Approximately ⅔ of all staphylococci isolated from clinical specimens were resistant to both penicillin and tetracycline They have been classified as "hospital" strains Resistance to chloramphenicol was present in just over 50 per cent Resistance to methicillin was not observed As most strains were represented by rather small materials, resistance patterns were determined only for staphylococci which were commonly found in clinical specimens Antibiograms for 75 77, Thorax BP and Thorax I strains are given in Table 7

TABLE 7

Relative Percentage of Resistance to Antibiotics in 3 Common Disease Producing Strains

Phage pattern	Resistant strains %				
	Pc	Er	Tc	Ch	Mc
75/77	95	62	55	68	0
Thorax BP	78	75	92	72	0
Thorax I	72	63	81	33	0

75 77 This homogeneous strain, believed on epidemiological grounds to have a common origin, showed striking individual variations in resistance to erythromycin and tetracycline There were 2 "variants one was present almost continuously in the unit up to summer closure the other was brought there in the autumn by carriers from other parts of the hospital The former was highly resistant to all antibiotics except methicillin, whereas the latter was usually sensitive to erythromycin and tetracycline, sometimes also to chloramphenicol

Thorax BP This strain gradually grew more resistant, or sensitive isolates became infrequent, the great majority were of intermediary resistance

Thorax I When introduced into the unit this strain was resistant to penicillin and tetracycline, and sensitive to erythromycin and chloramphenicol Within a month erythromycin resistant isolates were common

3 *Source of Staphylococci*

Extensive studies were undertaken to determine when and where pathogenic staphylococci were acquired As a detailed account will be published separately, the results shall be summarized briefly Of 41 infected patients, 14 (34 2 per cent) were carriers of the infecting strain already at the time of operation Contamination of tissues may have taken place either in the theatre *or* in the intensive-care ward They have been classified as *endogenous* (autoinfections) Infections that were regarded as *exogenous theatre acquired* developed in 4 patients (9 8 per cent) Among the remainder 22 (53 7 per cent) were acquired in the intensive care ward and 1 (2 4 per cent) in the nursing wards They have been classified as *exogenous, ward acquired*

In 23 tracheotomized patients 10 infections (43 5 per cent) were classified as endogenous 1 (4 4 per cent) as exogenous theatre acquired and 12 (52 2 per cent) as exogenous ward acquired

In 32 septic cases (78 1 per cent) human beings were established as the ultimate source of disease producing staphylococci

DISCUSSION

In 1962 the over all infection rate was 18 5 per cent and the staphylococcal infection rate 13 1 per cent These rates were higher than in 1961 when they comprised 13 9 and 10 6 per cent But the rise was in all probability an artefact due to closer surveillance in 1962 The accuracy of wound healing cards as the single source of information was shown to be no better than 65 per cent and that of bacteriological records 74 per cent As there was not complete overlapping of information supplied by these two sources their combined use detected about 80 per cent of all infections A time consuming review of the records of all operated patients added another 12 unreported infections Obviously 1 of every 5 infections would have remained unknown had not the control been included in the method of surveillance

Apart from the review of case records the criteria of infection had shifted In earlier reports there had been the bias of purely bacteriological criteria whereas the final decision whether or not a patient was to be classified as infected was now made by a surgeon Bacteriological verification was possible in about 74 per cent of all septic lesions Unfortunately the clinical suspicion of septicaemia was hard to verify Routine procedures failed to confirm the clinical diagnosis in 10 of 17 patients all of whom were receiving massive doses of antibiotics frequently through a catheter in the superior vena cava It is

recognized that these unconfirmed cases were not necessarily septicaemias but could have been due to other causes including toxic reactions to antibiotics and the postpericardiotomy syndrome

The clinical importance of postoperative staphylococcal disease is obvious from the high proportion of serious infections 35 were classified as severe 19 died Although infection contributed to the fatal outcome in many cases its importance could not be determined precisely since both infection and subsequent death occurred primarily in patients with serious underlying disease The immediate cause of death was most often failure of the cardiorespiratory system Most significant was that the number of serious staphylococcal infections had increased from 14 in 1961 to 35 in 1962 *i e* a rise of 150 per cent whereas the mortality in such cases remained at about 50 per cent

Taken as a group operations on the heart and great vessels carried a higher risk of postoperative infections than pulmonary operations the incidence of septic complications being 22 9 per cent in the former as compared to 12 5 per cent in the latter *Detailed analysis of the patients* their underlying illness and type of operation revealed however that this difference was not due to any specific vulnerability of the heart to infections

By grouping cardiac patients according to the nature of the underlying disease and the type of surgical procedure it became evident that the infection attack rate ranged from 0 to 53 5 per cent Whereas mitral valvulotomy was never complicated by infection sepsis developed in every second patient with a Starr prosthesis Similarly the rate ranged from 0 per cent in segmental resection to 27 3 per cent in pulmonectomy Obviously the risk of sepsis was related to factors inherent in the patient and to his operation

Clinically the nature of the infection also varied from group to group Patients undergoing prosthetic replacement of diseased heart valves were particularly prone to progressive infections such as tracheobronchitis and/or bronchopneumonia a deep abscess and septicaemia whereas other cardiac patients were subject to circumscribed lesions A similar difference characterized pulmonectomized and lobectomized patients Tracheobronchitis and/or bronchopneumonia empyema and a septic wound developed in the former in contrast to the latter in whom trivial septic wounds appeared

A comparison of sepsis rates for open and closed cardiac operations 38 5 and 7 5 per cent respectively showed that the difference was significant (p = 0 05) But this difference was influenced by numerous factors other than the type of surgical procedure which was used to define the two groups Thus the risk of sepsis was significantly higher in patients with enlarged hearts reduced cardiac reserve and frequently also secondary pulmonary changes They underwent prolonged intracardial operations made possible by extracorporeal circulation which is known sometimes to cause harmful side effects Their inci

sions were wide, foreign bodies were implant

Viable staphylococci in numbers sufficient t infection reached a susceptible site in every Starr prosthesis, but not in patients underg Therefore, the risk of staphylococcal sepsis pattern, was influenced not only by the presen invade the tissues, but by the sum of a large concept is supported by Lidwell's statistics P H L S investigation, where he demonstr procedures in themselves carry a low basic ri When associated with certain adverse factors cantly, it increased *multiplicatively* when coexisted

The small somewhat heterogenous material evaluate the association between the rate of

(*Lidwell* 1961, *Barnes* 1962) An age risk observed It was evident in patients on whom performed Pulmonectomized patients were, older than lobectomized patients More sign haps, that the case histories of the former "atypical" pulmonary infection prior to surg patients had been tracheotomized because res anticipated postoperatively If age was a det was lost among the effects of many other fact the septic outcome

Operations associated with high rates of lasting for more than 2 5 hours About one by infection had lasted for 2 5–3 5 hours hours There was an accumulation of lung range and of open heart operations in the of comparable operations, infected and non *a direct time risk relation Although of* simil tomy (3 3 hours) and lobectomy (3 5 hour strikingly different infection attack rates (cent) Still longer operations such as valve duration 5 2 hours), carried extremely high surgery became more extensive, host factors status—seemed to become increasingly impor sepsis Again, tracheostomy was a common

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infected patients The time-risk relation, if present, was also obscured by other factors

Postoperative septic complications were observed in a total of 34 among 80 tracheotomized patients Tracheostomy was a common denominator in 58 6 per cent of all infections Its epidemiological rôle could be established by the high incidence of patients who were colonized by prevalent staphylococcal strains By-passing the protective function of the nasopharynx, staphylococci from the air, adjacent sites of wound infection or contaminated suction catheters colonized traumatized mucous membranes Particularly infection-prone were tracheotomized patients with a median sternotomy, in whom slow-healing single-wound cavities frequently appeared

As a rule, the staphylococcus seemed to need an anatomical lesion to by-pass normal defense barriers Remaining *in situ* for 3–7 days postoperatively in most open-heart operations, a cannula, a vena cava catheter and several drainage tubes presented ambient staphylococci with 4–5 pathways by which they could gain access to intrathoracic structures in close proximity to foreign bodies, such as a valve prosthesis and an aortic or myocardial graft

Open pathways may also help to explain why approximately 50 per cent of all infections were due to ward-acquired, exogenous staphylococci, and why endogenous staphylococci were responsible for close to 50 per cent of infections in those carriers who underwent open-heart surgery There were, in fact, outbreaks of autoinfection among infection-prone patients during periods when exposure to epidemic strains was infrequent or of short duration (*Lindbom & Laurell*) Whatever the mechanism, certain operations seemed to induce in the patient a state of increased susceptibility to his own staphylococci This is all more noteworthy as there was only a single primary infection – a case of *Klebsiella* bronchopneumonia – that could be attributed to endogenous gram-negative bacteria

Staph aureus was the predominating organism, responsible for more than 3 times as many monobacterial infections as *Staph albus* and 25 times as many as gram negative bacteria Although of secondary importance, *Staph albus* caused serious monobacterial infections in 3 patients who had a diseased valve replaced by a prosthesis Compared to *Staph aureus*, other organisms seemed to have less ability actually to initiate sepsis, even in infection prone patients As far as gram-negative bacteria are concerned their rôle was primarily that of a secondary invader in cardiac patients receiving methicillin and in certain tracheotomized lung patients, or of a contaminant in some trivial wound infections

Phage typing revealed that 5 strains of *Staph aureus* produced 75 6 per cent of all infections In contrast to 1961, staphylococci belonging to the 52 52A/80 81 complex played a minor rôle, whereas staphylococci identified by a clear cut 75/77 pattern remained entrenched in the

unit, or were repeatedly reintroduced from other reservoirs As only about 40 per cent of all staphylococci were typable at RTD by the basic international set of phages patterns were often highly complex Particularly hard to interpret were two strains, characterized by wide patterns involving phages of groups I and III One, Thorax BP, was lysed at RTD × 1000 by phages 52, 52A, 80, KS6 7, 42E, 53, 54 75 77 and 83A The other, Thorax I had a fairly stable pattern of inhibition with phages 81, KS6 47, 83 and, less commonly, 52, 52A, 7, 42E and 75 When present simultaneously in the unit, they were frequently hard to differentiate Initially, this could be done with certainty only by titration

Antibiotics were important determinants of the types of organisms present in the unit Approximately 60 per cent of the patients harboured staphylococci resistant to penicillin, tetracycline and chloramphenicol When patients received large doses of a single antibiotic or multiple drugs in combination, they acquired resistant hospital staphylococci rapidly and with amazing frequency, despite nasal chemotherapy

Two tetracycline resistant staphylococcal strains merit special attention Isolated originally from patients with staphylococcal diarrhoea they were shown to produce enterotoxin B (*Hallander* 1965) They were identified by the 75/77 and Thorax I patterns Clinically, they caused a multiform disease that included one or more of the following symptoms a septic wound bronchopneumonia diarrhoea and septicaemia Diarrhoea occurred in ⅓ of the patients, only rarely was it the sole sign of infection These strains seemed to have a special affinity to mucous membranes and the ability to induce profuse secretion of sputa or stools resulting in massive environmental contamination There was no evidence that infection prone patients were predisposed to complications by such strains, although tracheotomized patients played a central rôle in a vicious epidemic circle either as victims or disseminators or both

In roughly ¾ of all infections, a person most commonly a patient could be established as the ultimate source of disease producing staphylococci It was evident that factors inherent in the patient and his operation were important determinants of whether a given staphylococcus in a given patient was to cause disease It was nevertheless equally obvious that numerous external factors – such as structure of the unit, intramural movements of the patients and rate of turnover contributed to the creation of a [illegible] a source where characteristics and prevalence of staphylo[illegible] influenced the epidemiological pattern

SUMMARY

Postoperative septic complications developed in a total of 58 patients (18 5 per cent) *Staph aureus* was the predominating organism ac

counting for 41 infections About ⅓ were due to endogenous staphylococci frequently acquired in hospital but before admission to the thoracic surgery unit Thirty-five infections were serious, contributing to the fatal outcome in 19 cases Enterotoxin B producing staphylococci played an important rôle

Intrathoracic operations were associated with wide variations in the specific infection attack rate, which ranged from 0 per cent after mitral valvulotomy to 53 5 per cent after prosthetic replacement of diseased heart valves The incidence of postoperative sepsis showed a positive correlation to numerous non-bacterial factors, such as underlying disease functional state of heart and lungs, type of surgical procedure, postoperative tracheostomy, indwelling catheters in the superior vena cava and multiple drainage tubes These factors were primary determinants of whether a given staphylococcus in a given patient was to cause disease They also explain periodic outbreaks of non-epidemic infections due to endogenous staphylococci Numerous other factors determined which types of staphylococci patients were exposed to

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STUDIES ON THE EPIDEMIOLOGY OF STAPHYLOCOCCAL INFECTIONS

4 *Effect of Nasal Chemotherapy on Carrier State in Patients and on Postoperative Sepsis*

By

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Received 22 vii 66

Recent studies of hospital infections caused by *Staph aureus* have emphasized that carriers play a central rôle in the epidemiological cycle, either as victims, or sources, or both The association between nasal carriage and an increased risk of postoperative sepsis was first described by *Williams et al* (1959) Later, *Ketcham et al* (1962) presented evidence correlating the sepsis rate after radical cancer surgery to the carrier status of the patients Our previous studies of patients undergoing thoracic surgery confirmed this correlation Thus, roughly 1/3 of those in whom postoperative staphylococcal sepsis developed were infected by staphylococci identical to those carried preoperatively in the anterior nares (*Lindbom* 1964, *Lindbom & Laurell* 1967) These reports indicate that endogenous staphylococci may be responsible for a variable proportion of septic complications in nasal carriers

White (1961) showed by semi-quantitative studies that a correlation exists between skin carriage and heavy nasal carriage Staphylococci could be recovered from only 5 per cent of skin cultures in patients who were carriers of less than 100 000 colonies per nasal swab, whereas the corresponding incidence in patients with more than 100 000 colonies per swab was 44 per cent

On the assumption that the nasopha[illegible]ynx is the main human reservoir, its elimination as a source of inf[illegible]ction would be a logical prophylactic measure to minimize the r[illegible] of autoinfection and, consequently, to reduce the over-all ris[illegible] sepsis *Delafield et al* (1941) [illegible] nasal reservoir directly by local [illegible] Subsequently, various methods [illegible] [illegible]ction have been used, the commonest being a cream containing n[illegible]mycin and chlorhexidine applied [illegible] intervals to the nostrils Better re[illegible]ults were obtained with a nasal s[illegible] containing gramicidin and framyc[illegible]tin (*Stratford et al* 1960 [illegible]

methicillin ointment (*Varga & White* 1961) and by spraying the ward air with methicillin (*Elek & Fleming* 1960)

Nasal chemotherapy was therefore instituted in The Department of Thoracic Surgery as a preventive measure to be used by all patients 'Naseptin" was chosen for preoperative prophylaxis, whereas "Sofra mycin" was chosen for eradicative treatment and for postoperative use, partly because it could be used in postoperative patients with a mini mum of disturbance, in view of its mode of application

The following four criteria were used to assess the efficiency of nasal chemotherapy (1) Influence on the carrier rate of the popula tion at risk (2) Influence on the carrier state of individual patients (3) Influence on the incidence of autoinfection (4) Influence on the ecology of epidemic strains Since they were parameters of different aspects of the host-parasite relation, they provided means of cross validation

MATERIAL AND METHODS

Characteristics of the patients method of surveillance antibiotic policy, arrange ment of the wards and basic bacteriological technique have been described elsewhere (*Lindbom et al* 1967) The epidemiological survey included extensive carrier exa minations and standardized environmental studies in the intensive-care ward

Bacteriological Routine

Nasal and throat swabs were taken from patients on the day of admission or as soon after as possible They were also swabbed on the day of operation and daily during the postoperative phase in the intensive-care ward except on Saturdays Sundays and Mondays The study is based on totally 2736 nasopharyngeal cultures *from which Staph aureus was isolated on 903 occasions The disc diffusion method* was used for sensitivity determinations (*Ericsson et al* 1954) All coagulase positive staphylococci were phage typed by the technique of *Blair & Wil*— [illegible]

Nasal Chemotherapy On admission all patients [illegible] fectant cream (Naseptin®), con[illegible] *SO_4 0 5 per cent* They were [illegible] times daily In general patie[illegible] having been informed of the aim of the trial

All patients found to be carriers on admission in addition to postoperative pa tients in the intensive care ward were supplied with a nasal spray (Soframycin®) containing framycetin 1 25 per cent gramicidin 0 005 per cent and metaoxidrine 2 5 per cent The nebulizer was to be squeezed sharply into each nostril and the pharynx 3 times daily

On their return to the nursing wards patients received a second tube of nasal cream

A source of error common to all swabbing of patients undergoing nasal chemo therapy was the transfer of an antibacterial substance from the mucous membrane to the swab This carry over effect could not be excluded but the risk was expected to be lowest immediately before the first treatment of the day Consequently when ever possible nasopharyngeal samples were taken in the morning

Definitions Patients were classified as persistent carriers when more than three cultures were positive They could be divided into two groups those who carried the same phage type repeatedly and those who changed types frequently Persistent non-carriers were patients from whom more than three negative cultures were obtained The remainder were classified as intermittent carriers

Since both nose and throat swabs were used the presence of staphylococci at either site was regarded as evidence of carriage at the time of swabbing

BACKGROUND INFORMATION

The study took place from January 1st to December 31st, 1962, inclusive Naso pharyngeal cultures were obtained from 402 patients, 313 of them had been operated on Postoperative infection developed in a total of 58 patients *Staph aureus* was isolated from septic complications in 41 (13 1 per cent) of these cases

The pathogenesis of staphylococcal infections clearly depended to a considerable extent on host susceptibility Infection attack rates ranged from 0 to 53 5 per cent High rates were associated with multiple adverse factors Taken as a group opera tions on the heart and great vessels carried a higher risk of sepsis than pulmonary surgery In the former the over all rate was 22 9 per cent, ranging from 7 5 per cent after certain closed cardiac operations to 35 8 per cent after open heart procedures Septic complications occurred in 12 5 per cent of the patients who underwent pulmo nary operations The infection attack rate was 27 3 per cent after pulmonectomy and 8 3 per cent after lobectomy

Hospitalization in the unit lasted for 29 5 days for the "average" surgical patient, of this time he spent 9 5 days awaiting an operation lasting for 3 3 hours, and 4 5 days in the intensive care ward Due to factors inherent in the patient/operating complex, there were marked variations in the duration of these phases (*Lindbom et al* 1967)

Data on phases of hospitalization mode of nasal chemotherapy and nasopharyn geal examinations are summarized in Fig 1

Fig 1

Phases of Hospitalization, Mode of Nasal Chemotherapy and Nasopharyngeal Carrier Examination

Environment	Nursing ward		Intensive care ward			Nursing ward
Average duration	9 5 days		4.5 days			15 5 days
Nasal chemotherapy Non carrier Carrier	 Naseptin Naseptin Soframycin		 Soframycin Soframycin			 Naseptin Naseptin
Carrier examination	↑ A	↑ O	↑ PO	↑ PO	↑ PO*	

* Arrows indicate nasopharyngeal examinations on admission (A) at operation (O) and subsequently in the intensive care ward (PO)

RESULTS

Carrier Rate

On admission, a total of 142 out of 402 patients examined (35 3 per cent) were found to be nasopharyngeal carriers A second culture was [illegible] ion, which implies that the [illegible] was studies in 85 per cent [illegible] as 41 4 per cent on admission and 26 7 per cent at operation Th[illegible] degree of reduction was related to certain host factors such as ag[illegible] underlying disease, previous hospitalization and antibiotic treatme[illegible] In 29 children, the carrier rate was reduced from 31 2 to 15 9 per cent In 27 patients with pulmonary in fection the rate fell from 43,7 to [illegible] 7 per cent In contrast to the child ren, the latter had been hospitalized for long periods before the current

admission owing to the severity and chronicity of the underlying illness The majority had also received several courses of systemic antibiotic treatment

Two preoperative and two postoperative cultures were collected from 146 patients i e the influence of *prolonged* nasal chemotherapy (>2 weeks) was studied in 46 7 per cent of the operated population In this group the rate of carriage was 41 8 per cent on admission fell to 24 7 per cent at operation but rose to 43 8 per cent on the 2nd and 3rd postoperative day despite nasal chemotherapy Later, the rate of carriage was established at nearly 50 per cent It should be noted however that the number of cultures per patient was dependent on the time spent in the intensive care ward Little information is therefore available about the carrier state of patients undergoing operations necessitating only a brief period of intensive postoperative supervision whereas the bulk of information deals with patients on whom extensive operations had been performed The former group including resection of aortic coarctation and lobectomy spent an average 2 3 days in the intensive care ward This contrasted sharply to the latter group (replacement of diseased cardiac valves and pulmonectomy) who remained there for 6–11 days

Antibiograms Approximately 50 per cent of all nasopharyngeal staphylococci were resistant to penicillin and tetracycline They were classified as hospital staphylococci During the study 7 4 per cent of the strains tested were found to be resistant to neomycin

Carrier State

Conclusions on the individual state of carriage in 146 patients were based upon sequences of four nasopharyngeal cultures

Positive cultures on admission This group consisted of 61 patients In 16 of them staphylococci were also isolated from the three following specimens In 15 patients the initial positive culture was followed by three negative ones The remainder (30 patients) were intermittent 11 yielded two positive cultures 19 only one positive culture

Negative culture on admission This group consisted of 85 patients 49 of whom remained negative Ten patients were converted into a positive state and staphylococci were isolated from the three following specimens Of the remainder (26 patients) 11 yielded two positive cultures and 15 only one

In Table 1 the patients are classified on the basis of the number of positive cultures without reference to their order It is seen that 45 (30 8 per cent) were persistent carriers 22 (15 1 per cent) intermittent carriers and 79 (54 1 per cent) persistent non carriers

Fragmentary evidence—based on the *trend* observed in sequences of 5 or 6 cultures obtained from patients in the intensive care ward for a prolonged period—showed that the proportion of persistently positive

carriers rose from approximately 30 per cent on the 2nd postoperative day to 50 per cent on the 5th A large part of this increase could be traced to intermittent carriers

Phage typing of staphylococci recovered from patients undergoing nasal chemotherapy permitted an analysis of its effect on individual carriers As 5 cultures were regarded as a minimum sample, only 86 carriers were available for analysis It was found that 33 per cent of the patients retained the strains present on admission whereas 22 per cent lost them Hospital staphylococci were isolated transiently from 28 per cent and persistently from 17 per cent

TABLE 1

Patterns of Carriage in 146 Patients as Determined by 4 Consecutive Nasopharyngeal Cultures

Classification	Pattern of carriage		No of		Sepsis rate
	Positive	Negative	Patients	Infections	%
Persistent carriers	4	0	16	11	68 8
	3	1	29	10	34 5
Intermittent carrier	2	2	22	4	18.2
Persistent non carriers	1	3	30	5	16 7
	0	4	49	11	22.5
			146	41	13 1

Relation between Carrier State and Rate of Sepsis

It is also evident from Table 1 that a positive correlation existed between the number of positive specimens and the rate of sepsis It rose from 16 7 per cent in patients with one positive culture to 68 8 per cent in those with four Obviously persistent non carriage did not preclude sepsis as postoperative infection occurred in 22 5 per cent of the patients from whom four negative specimens were obtained

The rate of sepsis was 46 7 per cent in persistent carriers 18 2 per cent in intermittent carriers and 20 3 in persistent non-carriers To illustrate the clinical importance of the carrier state in 41 infected patients the acquisition of disease producing staphylococci is summarized in Table 2 Details of this epidemiological study will be presented in the following paper (*Lindbom & Laurell* 1967) Fourteen infections (34.2 per cent) occurring in persistent carriers were classified as endogenous They were caused by strain[illegible] carri[illegible]d on admission or acquired preoperatively in the ward Twenty s[illegible]ven infections were classified as exogenous They occurred in all t[illegible] of carriers Four infections (9 8 per cent) were acquired in the the[illegible] 22 [illegible]1 3 per cent) in the intensive-care ward and 1 (2 4 per cent in the ward

Persistent carriers could be divided into two groups, *i e* 9 carriers of a single strain, and 12 carriers of multiple strains Five of the latter

acquired their disease producing strains preoperatively 1 during surgery and 6 postoperatively in the intensive care ward In persistent non carriers 2 infections were theatre acquired and 14 ward acquired

TABLE 2

Summary of Data in 41 Cases of Staphylococcal Sepsis

State of carriage	Acquisition of disease producing staphylococci: before admission	Ward	Op theatre	Intensive care ward	Ward	
Persistent (single strain)	[illegible]					9
Persistent (multiple strains)	3	2	1	6		1[illegible]
Intermittent			1	3		4
Non carriage			2	13	1	1[illegible]
	12	[illegible]	4	2[illegible]	1	

———— endogenous infection (boxed in table)
exogenous infection

Taken as group persistent carriers were comparable with regard to patterns of carriage although they differed individually with respect to strains carried and various patient factors Analysis of carriers and non carriers infected and non infected revealed that patients in whom sepsis developed had generally undergone extensive surgical procedures such as certain types of open heart operation and pulmonectomy that were associated with multiple adverse factors Thus infection prone persistent carriers tended to contract endogenous infections whereas persistent non carriers contracted exogenous infections when exposed to certain strains prevalent in the environment

DISCUSSION

The effects of nasal chemotherapy reported by various authors have been difficult to evaluate in view of the lack of generally accepted criteria The index of success has often been calculated from the difference between pre and post treatment cultures Intermittent carriers sometimes occurring in up to 60 per cent of a population (*Hutchinson et al* 1957) can be expected to give rise to a certain number of false negative results In the hands of *Bassel et al* (1963) the reliability of a single swab was 80 per cent This implies that in the absence of frequent swabbings intermittent carriers might be incorrectly classified as successfully treated Persistence of staphylococci in the nose despite chemotherapy must also be differentiated from the acquisition of new strains It might have been ideal if the carrier state of patients

undergoing treatment had been known before it was started, but this was not practicable in the present study Instead, the pattern of carriage had to be determined during therapy The use of controls was ruled out because the nasal carrier state was unknown, and the population was too small and too heterogeneous for its variations to be equalized by random sampling

In patients who were to be operated on, the carrier rate on admission was 41 4 per cent, i e, well within the 35–50 per cent range normal for adults Although hospitalization generally increases the proportion of persons carrying hospital staphylococci and tends to produce an over-all rise, nasal chemotherapy actually reduced carriage during the pre-operative phase by about 40 per cent This initial decrease was counter-balanced within 1–2 days of operation by an equal increase, despite continued treatment There are several possible explanations of this unexpected rise The simplest one is the presence of neomycin-framycetin-resistant strains which rapidly colonized postoperative patients This was not, however, the case as only a fraction (7 4 per cent) were resistant Alternatively, Soframycin spray may have had a less protective effect than Naseptin cream, although this would be at variance with the results of *Stratford* and co-workers (1960), who found Soframycin to be superior to Naseptin and claimed 100 per cent success in treatment It is conceivable that nasal chemotherapy was, of necessity, less than adequately applied during the early, hectic postoperative phase in patients with urgent circulatory or ventilatory problems This possibility cannot be disregarded Yet, the disproportionate frequency of persistent non carriers in the population at risk (50 per cent) seems to be attributable to fairly strict nasal chemotherapy

Since nasal chemotherapy caused a preoperative decrease in the over-all carrier rate, but failed to prevent a postoperative increase, it is obvious that environmental conditions differed

The epidemiological study, of which the results will be reported in the following paper (*Lindbom & Laurell* 1967) showed that certain patients—disseminators—caused massive, widespread environmental contamination in the intensive-care ward This ward was a bottle-neck, where newly operated patients in clos[illegible] proximity were often exposed to massive doses of disseminated staphyloco[illegible] In contrast, only a fraction of newly admitted patients [illegible] re exp[illegible]d to disseminators in the nursing ward

Nasal chemotherapy had littl[illegible] individual pattern of carriage It did not materially r[illegible] the [illegible]portion of persistent carriers, which remained at about [illegible] Phage typing and antibiograms revealed that 22 per cen[illegible] the [illegible]nts lost staphylococci carried on admission while 33 [illegible] them The rapid postoperative increase in the carr[illegible] rat[illegible]urred mainly in intermitt[illegible] carriers Hospital staphyloc[illegible] [illegible]lated transiently from [illegible] cent of the patients and persistentl[illegible]m 17 per cent

The present results support the view of *Williams et al* (1959) that a relation exists bewteen staphylococcal carriage and an increased risk of sepsis The rate of sepsis rose from 16 7 per cent in patients with one positive culture to 68 8 per cent in those with four Sepsis was 2 3 times as common in persistent carriers as in persistent non carriers Obviously, no direct causal relation existed since sepsis developed in 1 of 5 non carriers

A characteristic of the population at risk was that endogenous staphylococci accounted for 34 2 per cent of all infections They were particularly common in patients on whom valve replacement operation had been performed and were responsible for every second case of sepsis (*Lindbom et al* 1967) Thus, the risk of sepsis was dependent not only on the carrier status but also on numerous factors inherent in the patient and his operation, such as underlying disease, functional state of heart and lungs, type of surgical procedure and postoperative tracheostomy

But it was equally obvious that environmental factors were of importance (*Lindbom & Laurell* 1967) Exogenous staphylococci acquired in the intensive care ward accounted for 51 3 per cent of all postoperative infections Consequently, staphylococci carried by the patient at operation or acquired postoperatively caused 85 5 per cent of all infections

SUMMARY

Under the existing conditions nasal chemotherapy neither eradicated nor suppressed staphylococci carried persistently in the nasopharynx Nor did it protect intermittent carriers from the implantation of virulent hospital staphylococci The ecology could not be influenced by this measure, and the incidence of infection was not reduced

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STUDIES ON THE EPIDEMIOLOGY OF STAPHYLOCOCCAL INFECTIONS

5. *Importance of Environmental Factors During the Early Postoperative Phase*

By

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Received 22.xii.66

In a previous study which dealt primarily with clinical aspects, intrathoracic operations were found to be associated with a wide variation in the specific infection attack rate, which ranged from 0–53.5 per cent (*Lindbom et al.* 1967). In a heterogeneous population the incidence of postoperative sepsis showed a positive correlation to numerous patient factors, such as underlying disease, functional state of heart and lungs, type of surgical procedure, postoperative tracheostomy, indwelling catheters in the superior vena cava and multiple drainage tubes. Such factors were primary determinants of whether a given staphylococcus in a given patient was to cause disease. Infection-prone cardiac patients were found to contract sepsis even when staphylococci of known virulence were absent from the unit. Indeed, there were outbreaks of non-epidemic staphylococcal disease caused by endogenous staphylococci. The importance of the staphylococcal carrier state has been described separately in Part 4 (*Lindbom & Laurell* 1967). Sepsis developed in about 50 per cent of all persistent carriers who were in need of prolonged postoperative supervision because of circulatory and respiratory problems. But it was equally obvious that concurrent environmental factors, such as structure of the unit, turnover and intramural movement of postoperative patients contributed to the creation of a common source reservoir where patients were exposed to staphylococci that varied continuously both quantitatively and qualitatively.

To determine the influence of environmental staphylococci on the relation between a patient and his surroundings, studies were undertaken to determine *when* and *where* pathogenic staphylococci were acquired by patients in whom sepsis developed, and *how* epidemic strains were disseminated.

MATERIAL AND METHODS

Characteristics of the patients, definition of terms method of surveillance, antibiotic policy, nasal chemotherapy, arrangement of the wards and basic bacteriological technique have been described elsewhere (*Lindbom et al* 1967) An account is given in the following of a comprehensive epidemiological survey, including regular carrier examinations, standardized environmental examinations in the intensive care ward and a pilot study of the operating theatre

Environmental investigation (a) Intensive care ward Standardized studies were performed each week from Tuesday to Friday inclusive They were focused on the patients and their immediate surrounding Consisting of 6–10 cultures, depending on the number of patients in the ward, each '*patient-environment unit*' included sampling of the nasopharynx, bedding, furniture, equipment, air and personal belongings

The sweep plate method' was used for sampling of bedclothes and other fabrics Bacterial contamination was sampled by rubbing the relevant surface with sterile cotton swabs wetted with broth 'Sedimentation plates' were used to examine simultaneously the air from several sites in the ward Standard Petri dishes con-

of the operating team At each operation, 10 plates were placed around the patient at predetermined sites and exposed for the duration of operation

environment units collected on 224 different days In addition 267 nasopharyngeal cultures were obtained from 77 members of the staff and personnel The study is based on a total of 2670 staphylococcal isolates specified in Table 1

TABLE 1
Background Information on Staph aureus *Isolated in the Thoracic Surgical Unit*

Sample obtained	Source	Material		No of strains
Routine work up	Patient	Clinical specimens		
		Wound infections	85	
		Blood	23	
		Faeces	13	
		Sputa bronchial secreta	89	210
Epidemiological study	Patient	Nasopharyngeal cultures		905
	Environment	Air/culture plates	659	
		Contaminated objects	204	863
	Personnel	Nasopharyngeal cultures		92

BACKGROUND INFORMATION

Detailed information about the patients their operations and infection attack rates was given in previous papers in this series (*Lindbom et al Lindbom & Laurell* 1967) These also contained data on phage patterns and antibiograms of disease producing staphylococci Consequently only information relevant to the epidemiological approach will be given here

The total number of operated patients was 313 The monthly average of operations per operating day varied from 1 1 1 3 exceptions being May–July, September and

December when it reached 17 The duration ranged from 12 96 hours (average 33 hours) The average patient remained 29 5 days in the unit the preoperative phase lasted for 9 5 days the postoperative supervision phase spent in the intensive care ward for 4 5 days Due to patient factors the duration of these phases varied considerably The need of supervision in particular was greatly influenced by patient/operation factors It ranged from 24 hours to 52 days

The intensive care ward was a small area divided into three communicating parts two 5 bed sections and one single bed emergency room There was a constant need for isolation facilities in this open ward which was the centre of the unit of postoperative supervision and specialized intensive care Highly susceptible patients with circulatory and ventilatory problems were nursed in close proximity for long periods In practice it functioned as a bottle neck through which most newly operated patients had to pass and affected the turnover of patients

Variations in the operating list influenced the population in the intensive care ward When several short operations were performed in one day, the need of post operative supervision was generally short The turnover of postoperative patients was rapid When several open heart operations were concentrated to a brief period turnover grew slow resulting in an accumulation of infection prone patients Consequently there were periods when the population at risk was composed of patients with low to moderate susceptibility alternating with periods when most patients were characterized by high susceptibility Exposure to the ward lasted for 1 2 days for low risk patients and 10–13 days for high risk patients

There were appreciable variations from month to month in number and types of operation Open heart surgery comprised 12 56 per cent of all operations averaging 33 per cent In October and November it made up 48 and 56 per cent respectively No direct relation could be discerned between fluctuations in the monthly rate of sepsis and total number of operations or their sum in hours

In early January when the study was started staphylococci of proven virulence—mainly strains 75/77 and variants of the 80 81 complex—were not uncommon in the nasopharynx of patients and personnel 15 per cent of the former including 1 disseminator of 75/77 staphylococci and 4 per cent of the latter were carriers Total summer closure in July almost completely disposed of such strains as they were found in less than 1 per cent of both patients and personnel in August

Classification of infections Infections were classified as endogenous or exogenous When a patient who was a carrier of a particular strain on the day of operation subsequently contracted a septic lesion it was clearly impossible to determine whether contamination actually took place in the theatre or in the wards but for epidemiological purposes classification of the infection as of *endogenous* orig n was equally relevant

Infections were defined as *exogenous theatre acquired* on the basis of the following criteria if a known nasopharyngeal carrier participated in the operation or was present in the "critical area of the theatre the lesion was diagnosed within 5 days of operation and the disease producing strain was identical to the carrier strain Other sources were disregarded except in a pilot study of 18 open heart operations that were monitored by sedimentation plates and nasopharyngeal cultures from all members of the team

If no known nasopharyngeal carrier of a strain that caused postoperative sepsis was present in the theatre and clinical bacteriological evidence implied that the strain had not been acquired until after operation contamination was assumed to have taken place postoperatively Such cases were denoted as *endogenous* or *exogenous ward acquired infections*

RESULTS

Epidemiology of Staphylococcal Disease

Postoperative infection developed in a total of 58 patients *Staph aureus* was isolated from septic complications in 41 of these Information about certain aspects of the situation is summarized in Fig 1 The monthly incidence of staphylococcal sepsis ranged from 0 29 6 per cent, with a cumulative incidence of 13 1 per cent

Up to summer closure, 19 of 33 infections were due to staphylococci. The maximal incidence, 17.2 per cent, was reached in May, when 5 open heart patients contracted sepsis, 3 were endogenous infections. As a whole, the situation was characterized by numerous cases of sporadic infection. Only 7 septic cases could be attributed to endemic staphylococci, 75/77 and a strain denoted as 'Thorax BP' (for phage pattern, see *Lindbom et al* 1967), which had been present in the unit for prolonged periods without creating epidemic conditions. Excluded from Fig 1 were 6 cases of ill defined respiratory tract symptoms associated with slight fever and copious sputum from which 75/77 staphylococci were recovered.

After summer closure, the staphylococcal sepsis rate rose to a maximum of 29.6 per cent in November. Twenty-two of 25 infections were caused by staphylococci. Two strains, 75/77 and 'Thorax I' (for phage pattern see *Lindbom et al* 1967), accounted for 15 infections, while sporadic infections made up a smaller proportion. Whereas Thorax I was present continuously over a long period, 75/77 staphylococci were repeatedly introduced into the unit by carriers from other parts of the hospital, in whom endogenous infections developed.

Partial closure in late December reduced the rate of sepsis to 12.5 per cent.

Sources of Staphylococcus

Staphylococcal Carriers Patients The over-all carrier rate on admission was 35.5 per cent. In a group previously hospitalized for long periods due to the severity and/or chronicity of the underlying illness, it was 41.8 per cent. About 15 per cent of the former and 45 per cent of the latter carried staphylococci resistant to both penicillin and tetracycline.

Nasal chemotherapy was used throughout the study in all patients. As a detailed account of its effect will be published separately, results will be summarized briefly.

Nasal chemotherapy resulted only in a temporary preoperative reduction of 40–50 per cent in the carrier rate. Within a few days of the operation the rate had returned to the level on admission. Postoperative changes in the nasopharyngeal flora were studied by repeated swabbings. In this selected group of long exposure patients about 33 per cent retained strains carried on admission. Hospital staphylococci acquired before and after operation were recovered from 16 and 56 per cent respectively. The latter were a transient finding in about 2/3 of the patients and persistent in 1/3. The incidence of consistently positive carriers rose from 30 per cent on the second postoperative day to 50 per cent on the fourth. As the need of prolonged supervision was closely related to the type of surgery, colonization was most common in patients with a valvular prosthesis.

Personnel The over-all carrier rate as determined in 77 persons was 34.6 per cent. Repeated nasopharyngeal cultures were obtained from 44 members of the permanent staff. Of these 25 per cent were persistent carriers, 34 per cent intermittent carriers and 41 per cent non carriers.

TABLE 2

Relative Incidence of Environmental Staphylococcal Strains Isolated in the Intensive Care Ward

Phage pattern	Relative incidence %												
	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	
80/81 complex	1	5	3	17	13	1			10	1	2	1	
6/7/47/53/75	12	2	14	2	9	20			7	5	2	3	
75/77	34	53	6			1			5	1	15	2	
RTD × 1000 6/7/47/53/75/77	7	1	17	20	6	1			8	7	5	9	
RTD × 1000 Thorax BP	1	1	1	15	15	44			25	16	4	2	
RTD × 1000 Thorax I								–		19	47	40	
RTD × 1000 NT	17	11	24	13	16	18			22	14	10	13	
Various other patterns at RTD × 1000	27	19	21	16	17	14			17	9	6	17	
Total incidence of *Staph aureus* typed at RTD × 1000	52	32	63	64	54	76			72	65	72	81	
Total monthly no of positive environmental isolates	35	75	66	88	96	46			115	69	199	74	863

The following staphylococcal strains were isolated from persistent carriers (a) physicians Thorax BP RTD × 1000 29/80 KS6 and RTD × 1000 52/KS6 (b) nurses 3A 75/77 and 6/7/47/53/54/75 (c) maids 81/KS6 3A Thorax BP (d) technicians RTD × 1000 53/42D

As about 2/3 of the personnel were persistent or intermittent carriers strains prevalent in the environment were frequently recovered from their nasopharyngeal swabs Heavy carriage was however uncommon and in about 3/4 enrichment cultures were necessary to recover staphylococci

The surgical team comprised no nasopharyngeal carriers of staphylococci belonging to the 52/52A/80/81 complex or characterized by the 75/77 or Thorax I pattern With the exception of strains Thorax BP and RTD × 1000 53/42D no staphylococci in the personnel could be matched with strains subsequently responsible for sepsis

Environmental Pool Operating theatre A pilot study of the theatre environment was performed during 18 heart operations from October to December inclusive Altogether 32 colonies of *Staph aureus* giving 8 distinct lytic patterns were recovered from sedimentation plates Three strains could be matched with staphylococci carried by persons present one strain Thorax BP was carried by 2 surgeons the other two were patient strains

Intensive Care Ward Results of standard environmental studies are summarized in Table 2 It shows the monthly incidence of some common strains as well as an estimate of the intensity of contamination Whereas the level of non typable staphylococci remained fairly close to 15 per cent throughout the year most strains showed marked cyclic

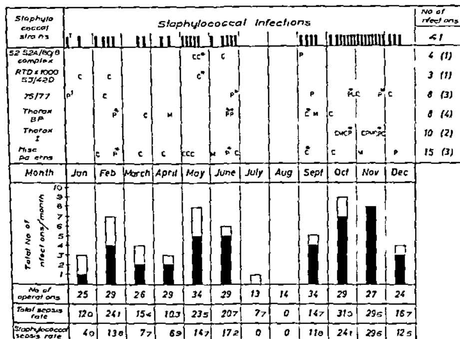


Fig 1

C Cardiac operation single strain of *Staph aureus*
C* Cardiac operation several strains of *Staph aureus*
P Pulmonary operation single strain of *Staph aureus*
P* Pulmonary operation several strains of *Staph aureus*
M Miscellaneous operations
Bracketed figures denote mixed staphylococcal infections
■ *Staph aureus* infections
□ Other bacterial infections

1 Operation performed in 1961 not included

variations, with steep increases and decreases The build up phase either preceded, coincided with or followed an accumulation of postoperative infections presented in Fig 1, depending upon such variables as degree of dissemination and average length of incubation period

Staphylococci characterized by the 75 77 pattern dominated the picture in January February, when they comprised 34 53 per cent of the pool Another rise, to 15 per cent occurred in November During April–June, a second strain, Thorax BP emerged as a common contaminator, reaching a maximum of 44 per cent in June from 25 per cent in September it fell slowly to a few per cent It was replaced by a third strain Thorax I, which dominated the pool in October December, reaching a maximum of 47 per cent in November

Staphylococci belonging to the 52 52A 80 81 complex formed a variegated group which comprised a small fraction of the pool during the greater part of the year Only in April May and September did the level exceed 10 per cent

TABLE 3

Classification and Comparison of Staphylococci from Different Sources by Phage Typing

A Patient nasopharyngeal strains

Group	Phage RTD	Dilution RTD × 1000	No of strains	%
I	81	67	148	16.4
II	53	14	67	7.4
III	225	178	403	44.5
I+III		214	214	23.7
NT		73	73	8.1
No of strains	359	546	905	
%	39.7	60.3		

B Personnel nasopharyngeal strains

Group	Phage RTD	Dilution RTD × 1000	No of strains	%
I	20	33	55	20.7
II	15	16	31	11.7
III	38	64	102	38.4
I+III		58	58	21.8
NT		22	22	8.3
No of strains	73	193	266	
%	27.5	72.6		

C Environmental strains

Group	Phage RTD	Dilution RTD × 1000	No of strains	%
I	51	24	75	8.7
II	41	22	63	7.3
III	256	130	386	44.7
I+III		251	251	29.0
NT		88	88	9.8
No of strains	348	515	863	
%	40.7	59.2		

Conformity between Different Sources

By way of comparison phage typing results relating to nasopharyngeal strains obtained from patients and personnel in addition to environmental strains are summarized in Table 3 They have been arranged by phage group and phage dilution

There was a close conformity in phage group distribution between staphylococci recovered from patients and their environment with the

single exception of group I staphylococci which were twice as frequent in the nasopharynx as in the surroundings This is in accordance with the observation that individuals whose strains changed were recolonized with such organisms as appear in the greatest number Although the general distribution of strains carried by the personnel was similar to that of patients it should be noted that groups I and II staphylococci were slightly more common among the personnel—who were not receiving nasal chemotherapy—than groups III and I + III which were less common The distribution showed relatively good conformity between all three sources only when strains were characterized by high indices of contamination *e g* groups III and I + III

Time and Place of Acquisition of Disease Producing Staphylococci

Endogenous Infections Autoinfections due to endogenous staphylococci developed altogether in 14 patients (34 2 per cent) 10 were due to strains carried in the nasopharynx on admission 2 were acquired in the unit before operation and the remaining 2 were isolated preoperatively from superficial skin pustules but not from the nasopharynx Phage typing disclosed that group III strains had produced 8 infections including 3 cases of 75/77 sepsis group II strains 4 infections and group I and group I + III strains one infection each

Exogenous Theatre Acquired Infections During the pilot study 7 patients contracted postoperative sepsis Only one infection a septic wound from which *Staph aureus* (Thorax BP) *Staph albus* and *E coli* were isolated fulfilled the criteria set up The others were either autoinfections or due to strains not present in the theatre *e g* Thorax I

During the whole year only 4 cases (9 8 per cent) were classified as exogenous theatre acquired Thus in addition to the Thorax BP infection mentioned there were 3 infections all due to staphylococci with the RTD × 1000 53 42D pattern which fulfilled the criteria There was only one known source of this strain in the unit a technician who was present during open heart operations subsequently complicated by infection

Endogenous or Exogenous Ward Acquired Infections The average interval between operation and recognition was 13 days Roughly 75 per cent of all infectious complications were diagnosed within 15 days 66 per cent between the 5th and 15th day

Intensive care ward Since the number of carriers increased with the time spent in this ward and the need of prolonged intensive supervision was closely related to the type of surgery nasal colonization was most frequent in patients who had undergone open heart surgery or pulmonectomy Strains identified as 75 77 Thorax BP and Thorax I were isolated from the nasopharynx of 63 patients It could not be established whether nasal colonization actually preceded wound contamination and caused endogenous infection occurred simultaneously

with inoculation of tissues by exogenous staphylococci, or was secondary to a septic complication For epidemiological purposes, the classification of 22 infections (53 7 per cent) as ward-acquired sufficed

The incidence of postoperative sepsis was 50 per cent in long-exposure patients from whom consistently positive nasopharyngeal cultures had been obtained, as compared to 18 8 per cent in consistent non-carriers

Nursing ward The epidemiological importance of the nursing wards was insufficiently studied Only one open-heart patient—a non carrier on discharge from the intensive-care ward on the 8th day—was believed to have acquired the contaminating 75/77 strain there It caused wound sepsis on the 16th day There was no known carrier among the personnel

Ecology of Staphylococci in the Intensive-Care Ward

The pool of staphylococci in the intensive-care ward was analysed by regular environmental studies The weekly number of distinct strains recovered from 'patient-environment units' was 5–15 They were usually present during short periods (2–3 days), which corresponded to the duration of intensive care for the average patient Only a few strains persisted in the ward

Relation between Patient and Inanimate Environment

A strain found in the nasopharynx of a patient could only rarely be matched with staphylococci recovered from his inanimate environment in the theatre or intensive-care ward

Although staphylococci generally could be isolated from a limited area surrounding an infected patient, contamination was rarely heavy as long as the lesion could be covered with adequate dressings Heavy contamination frequently accompanied cases of staphylococcal diarrhoea with profuse watery stools or a septic tracheostomy wound, with or without complicating tracheobronchitis and bronchopneumonia The presence of a disseminator was signalled by heavily contaminated air bedding and floor Within a day or two the strain was widely dispersed often isolated in the proximity of several non-carriers some of whom subsequently became nasal carriers The ward continued to be heavily contaminated until the patient was removed, when the strain disappeared within a couple of days

Of special interest were staphylococcal strains characterized as 75/77 Thorax I and Thorax BP Their epidemiological behaviour and ability to cause infection will be described in some detail

***Strain* 75 77** This strain was an easily identifiable marker' organism which has been endemic in many parts of the University Hospital since 1961 (*Lindbom* 1964) Characterized by a clear-cut 75 77 pattern both at RTD and RTD × 1000, strains from various parts of the hospital

differed mainly with respect to their resistance to tetracycline. Despite partial closure of the unit around the turn of the year 1961–62, they remained firmly entrenched there.

In December 1961, pulmonectomy had been carried out in a man with bronchial carcinoma. The postoperative course was complicated by by a bronchial fistula, empyema, and diarrhoea. Thoracoplasty on January 10th failed to arrest the infection. Bronchopneumonia and respiratory failure ensued. 75/77 staphylococci were isolated in large numbers from the sputum, bronchial secretion and faeces during a period of 35 days. He remained in the intensive-care ward for 8 days after the second operation. Being a copious disseminator, 75/77 staphylococci were recovered from his bedclothes, objects in his immediate environment, and air some distance away in the proximity of other patients, who were either non carriers or carriers of other strains. The 75/77 staphylococci were present *continuously* in the ward from the middle of January until the first week of March. As shown in Table 2, they comprised roughly 34 per cent, 53 per cent and less than 10 per cent of all positive environmental samples during this period.

No other cases of wound infection attributed to strain 75/77 occurred during this period of heavy environmental contamination. In June, a septic wound, classified as an auto-infection, developed in a patient who was a carrier of the strain already on admission. *Colonization* of the respiratory tract with 75/77 staphylococci was, however, a frequent phenomenon causing ill-defined infection of the respiratory tract or symptomless carriage. During the 8-week period when 54 patients were exposed to this massively contaminated environment 14 became colonized despite nasal chemotherapy. With few exceptions, these patients had been tracheotomized and had remained twice as long in the ward as the others (>7 days). Six patients subsequently ran a slight temperature, with concomitant profuse mucopurulent expectoration, from which 75/77 staphylococci were isolated. One patient—initially a non-carrier—on whom cardiac surgery had been performed, had purulent sputum and diarrhoea. Various postoperative complications necessitated a prolonged stay in the intensive care ward (31 days), where he was the main 'disseminator'. After his discharge in March, the strain rapidly became uncommon.

In the autumn 75/77 staphylococci were comparatively unusual in environmental cultures, with a relative incidence of 5 per cent or less, except in November, when 15 per [illegible] all staphylococci belonged to this strain. Nevertheless the rat[illegible] of 75/7[illegible] infection was higher than in the spring.

The first postoperative infecti[illegible]rred in September. A pulmonectomy performed on Septemb[illegible] was complicated by a bronchial fistula. Six nasopharyngeal cu[illegible]btained on admission and regularly afterwards, had been [illegible] staphylococci. On the 15th [illegible] day after the first reoperation [illegible] staphylococci were recovered for

the first time from the patient's nasopharynx At this time, the only known source in the whole unit including the operating team, was a nurse, a healthy persistent carrier of the strains for more than 1 year During the early postoperative phase she had helped to remove secreta from his airways by means of suction catheters Not until the 17th, when the strain was found in copious amounts of tracheobronchial secreta, could it also be recovered from the patient's immediate environment As he did not survive a second re-operation, performed 2 days later, the period of dissemination was short In tissues removed at autopsy, 75/77 staphylococci and *E coli* were present in large quantities Within 2 days of his death, 75/77 staphylococci were no longer recovered in the ward During a period of 4 days, when the strain had been present in the air and on objects in the immediate environment of this tracheotomized patient, 12 patients were exposed to it Single colonies were recovered from sedimentation plates in the vicinity of 3 patients, a few colonies were recovered once in a single culture from the nasopharynx of one

In October November, 75/77 staphylococci were responsible for postoperative sepsis in 4 patients Since 2 had been carriers of identical strains on admission, their infections were classified as auto-infections of the remaining 2 patients, 1 had acquired the strain in the ward before operation and 1 in the intensive care ward afterwards All patients had been tracheotomized, but only 2 began to disseminate staphylococci within a week of operation As they remained in the ward for only a short period thereafter, contamination was discontinuous and not extensive In total, 50 patients were exposed to 'showers' of 75/77 staphylococci The strain was isolated from sedimentation plates in the environment of 15 patients, and transiently from the nasopharynx of 5 patients

In December, a wound abscess from which 75 77 staphylococci were recovered appeared in one patient The diagnosis of septicaemia was considered but blood cultures remained negative The patient had acquired the strain after operation, presumably from an unknown source in the nursing ward

Strain Thorax I (RTD × 1000) This strain was not present in the unit until October 15th, when a carrier with ruptured chordae tendineae was admitted Open heart surgery was performed 2 days later Owing to complications mainly of a non-infectious nature he had to remain in the intensive care ward for 54 days A trivial infection of the tracheostomy wound and of the sternal incision developed on the 5th and 8th postoperative day, respectively Strain 'I' was recovered from a total of 121 samples obtained during 28 separate environmental studies, it was isolated from the nasopharynx, bronchial secreta, his bedclothes objects in the immediate surrounding, and the air

Being *continuously* present in the ward Thorax I strain was also recovered in the immediate environment of 28 patients *all of whom*

were non carriers or carriers of other staphylococci at the time of admission In October this strain was found in 19 per cent of all positive samples in November in 47 per cent and in December in 40 per cent Of 63 patients exposed to it in the intensive care ward 15 subsequently became colonized The time they spent there ranged from 2 10 days (average just over 6 days) Sepsis subsequently developed in 9 patients including 2 with a mixed 75 77 and Thorax I infection Again tracheostomy was the common denominator present in 7 patients Only 2 infections were trivial Septicaemia was suspected on clinical grounds in 5 patients all of whom died in 2 the diagnosis was confirmed at autopsy Empyema developed in the 2 remaining patients One patient with a perfectly healed wound after Beck s operation contracted staphylococcal diarrhoea

Strain Thorax BP (RTD × 1000) This was a fairly common organism in the intensive care ward (Table 2) and was recovered from the nasopharynx in 22 of 73 patients who had been exposed Heavy carriage was rare Over the period of investigation the antibiograms gradually changed Initially Thorax BP staphylococci were sensitive to antibiotics with the exception of penicillin In April variants resistant to tetracycline and chloramphenicol were noted together with sensitive strains Several months later sensitive strains had all but disappeared Obviously it cannot be stated unequivocally that strains from various human and inanimate sources were identical because of similarities in a common basic pattern Nevertheless there was a causal and temporal relation between several consecutively infected patients and a similarity in disease pattern that suggested the possibility of cross infection

In 8 patients trivial to moderately severe infections were diagnosed generally in the form of septic wounds They yielded a mixed flora of various staphylococcal strains including strain Thorax BP *Staph albus* and or enteric gram negative bacteria Characteristically Thorax BP was a secondary invader which did not cause a single case of mono bacterial infection There was one suspect case of septicaemia and none of diarrhoea Although isolated from sputa and bronchial secreta in several patients these were neither copious nor markedly purulent

Most of the time there was no recognizable source of Thorax BP staphylococci in the unit It was isolated only transiently from the nasopharynx of patients and personnel or recov red sporadically from air and inanimate objects There wer however 5 episodes of increased environmental contamination traceal to human sources In February cultures yield ng the BP strain c ob t ned from the blanket of a patient who had undergone surg for a traumatic rupture of the trachea 5 days earlier Within f d s it was recovered together with another staphylococcal str in f n sputa and purulent wound secretions Environmental cont nat on was insignificant A f weeks later it had colonized t k n of a patient with exacerb t n atopic dermatitis Appropriate d rm tolog cal treatment cleared up the

skin condition, and the postoperative course after pericardectomy was uneventful He nevertheless remained a moderate disseminator to bedding and ambient air A trivial sternal wound infection from which Thorax BP staphylococci and *E coli* were recovered subsequently developed in a tracheotomized fellow patient with a Starr prosthesis The upper respiratory tract was also colonized but he did not become a disseminator A third patient was colonized 2 weeks later, a few days after closure of an oesophago-bronchial fistula Repeated cultures of his sputa yielded BP staphylococci, but no septic complications appeared and environmental contamination was slight Throughout May the BP strain was recovered sporadically from sedimentation plates, but there was no known human source In June, a tracheotomized open-heart patient acquired the strain and became a moderately heavy disseminator, but his postoperative course remained uneventful Trivial wound infections later appeared in two pulmonectomized patients present in the ward In September, a minor auto infection occurred in an open-heart patient Environmental contamination was insignificant, but there was a second infection following excision of an aortic aneurysm in a patient who stayed simultaneously in the ward In October, one source of Thorax BP staphylococci could be traced a non-infected open-heart patient, who carried the strain before operation It was recovered sparingly from his nasopharynx, bedding and ambient air

DISCUSSION

The whole of 1962 was characterized by a fairly high incidence of staphylococcal disease i e, 41 cases (13 1 per cent) The monthly rate ranged from 0 to 30 per cent for 5 months it was <10 per cent, for another 5 months 10–20 per cent, and for 2 months 20 per cent The absence of septic complications occurred during the fortnight that preceded summer closure in July and that which immediately followed it in August, when the operating team was not complete and only short operations were performed There were no open heart operations and a single pulmonectomy The epidemiological importance of postoperative staphylococcal sepsis is obvious from the fact that it accounted for *between 50 and 100 per cent of all infections during 9 months of the* year it made up 66 per cent or more during 6 months

Most well defined infections were sporadic during the earlier part of the year Although short outbreaks of postoperative sepsis occurred most cases were due to strains causing single infections They seemed to bear a closer relation to a periodic accumulation of infection prone patients than to the presence of epidemic staphylococci The latter part of the year appeared to differ, since two strains 75 77 and Thorax I gave rise to numerous infections Despite these variations important epimiological similarities were observed

An earlier paper described the influence of hospitalization on patients

as reflected by changes in the carrier rate and the carrier state (*Lindbom & Laurell* 1967). An important selective factor was the routine use of prophylactic antibiotics. Given pre- and/or postoperatively they determined which types of staphylococci formed the hospital reservoir. At one time or another, approximately 60 per cent of the patients harboured strains resistant to penicillin, tetracycline and chloramphenicol. Patients receiving prophylactic antibiotics—either in large doses or in combination—acquired prevalent, multiresistant staphylococci with striking frequency and rapidity despite nasal chemotherapy. This is in agreement with the results of *Louria & Kaminsky* (1962), who demonstrated that the frequency with which patients harboured hospital staphylococci was related to both the kind and the amount of antibiotics administered. Actually, a combination of nasal chemotherapy and systemic prophylaxis failed to protect postoperative patients from either endogenous or exogenous staphylococci.

As to the origin of infecting staphylococci, they could be traced to human sources in 32 of 41 cases, whereas in 9 it remained unknown. The hospital staff seemed to play a less significant role in 1962 than in 1961 (*Lindbom* 1964). Only 5 patients were found to be infected by strains isolated earlier from personnel with whom they had been in contact. It is recognized, however, that nasopharyngeal swabs, obtained once every second month, fail to reveal carriage elsewhere (*e.g.* axillae, perineum, faeces, boils on unexposed skin) and may be insufficient, particularly in individuals who were intermittent carriers. Since the ability of nasal carriers to disseminate staphylococci seems to depend largely on high nasal counts (*White* 1961), the fact that it was necessary to use enrichment cultures to recover staphylococci from 3/4 of the staff suggests that their ability was low. Nonetheless, unknown sources among the staff may have been responsible for one or more of the 9 cases in which the origin of the infecting strain could not be ascertained. Altogether 27 infections could be attributed to staphylococci originating from patients. Frequent swabbings reduced the probability that unknown patient sources accounted for these additional infections.

Endogenous infections comprised more than 1/3 of the infections (14 cases). The infecting strain was isolated from the nasopharynx of 10 patients on admission and from skin pustules of 2 patients in the ward. Another 2 patients became nasopharyngeal carriers before operation. This experience emphasizes the clinical importance of the association between nasopharyngeal carriage and postoperative sepsis described by *Williams et al.* (1959). This is not, however, an universal experience, probably because of differences in ho[illegible]t operation factors. Tissue contamination may have taken place either in the theatre or postoperatively.

Of 27 exogenous infections only 4 were classified as theatre-acquired, *i.e.*, they could be ascribed to staphylococci carried by persons p[illegible]t in the theatre at operation. Unfortunately, sources other than naso-

pharyngeal carriers were disregarded, except during a pilot study of 18 open-heart operations, when the theatre was monitored by sedimentation plates Although results of a more extensive bacteriological work-up indicated that theatre-acquired infections were relatively rare, late septic complications may have been seeded by unknown carriers among the theatre staff That this must be regarded as a distinct but unproven possibility is also evident from an analysis of infections regarded as acquired outside the theatre

By definition, 23 exogenous infections were classified as ward-acquired, 22 were probably seeded in the intensive care ward and 1 in the nursing ward Whereas all cases fulfilled given criteria—i e, they were diagnosed more than 5 days postoperatively and could not be related to any known carriers in the theatre—only 13 could be related epidemiologically to known sources of staphylococci in the intensive-care ward In another 4 patients, discharge of pus from a superficial wound started more than 10 days after operation These septic wounds were regarded as infected most probably in the intensive-care ward Careful assessment, in each case, of clinical data and epidemiological evidence raised doubts as to the validity of the time limit as a means of distinguishing between theatre-acquired and ward-acquired infections This applied particularly when prophylactic antibiotics were given routinely Consequently, the place of infection remains in doubt for 5 exogenous infections

Even if no exact borderline could be drawn between sepsis acquired in the theatre and in the wards, it is obvious that the intensive care ward played a most important epidemiological role in the development of 18 infections (44 per cent) This confirmed our earlier conclusion that human sources, healthy or infected, were critical factors in the intensive-care ward, and frequently initiated vicious circles of cross-infection (*Lindbom* 1964) To test this conclusion, the interrelation was investigated between patients, their hospital environment and prevalent staphylococci

It was found that staphylococcal sepsis was the consequence of a complex set of factors, many of which were non-bacterial and unrelated to the intensive-care ward Some seemed to induce increased susceptibility, e g extensive surgery, extracorporeal circulation and implantation of foreign bodies Others provided portals of entry to bacteria, e g tracheostomy, slow-healing wounds and multiple drainage tubes Still others, such as operating list and need of prolonged supervision, sometimes created a high-risk postoperative population Finally, the structure of the unit influenced the risk of sepsis by bringing highly susceptible patients into close proximity, and exposing them to multiresistant staphylococcal strains prevalent in the *open* intensive-care ward

To determine the role of the intensive-care ward, standardized environmental studies were made They showed that staphylococci were

present continuously there but the composition of the flora varied both quantitatively and qualitatively Frequently, there were fairly abrupt changes which could usually be related to the introduction of a disseminator into the ward He produced massive contamination not only of his immediate surrounding but of the whole area Characteristically this patient had septic lesions which could not be covered adequately with dressings *e g* staphylococcal diarrhoea or a septic tracheostomy wound with or without further complications in the form of tracheobronchitis and bronchopneumonia It was especially noteworthy that—in the absence of cross infection—removal of a disseminator led to the disappearence of his strain from the ward within a couple of days Two staphylococcal strains in particular seemed to cause infections that predisposed to the status of disseminator These were staphylococci with clear cut 75 77 patterns or broad reactions of inhibition to group I and group III phages (Thorax I) Clinically they produced a multiform disease that included one or more of the following entities a septic wound tracheobronchitis bronchopneumonia septicaemia and enterocolitis Profuse diarrhoea occurred in about one third of the cases only rarely was it the sole symptom These strains seemed to have a special affinity for mucous membranes and the ability to induce massive secretion from the respiratory tract and or diarrhoea which led to massive contamination Using the technique of *Hallander* (1965) it could be shown that these strains produced enterotoxin B

Massive environmental contamination occurred periodically throughout the year First there was a phase of colonization followed by a second phase of sporadic infections primarily in susceptible patients Secondary disseminators perpetuated a vicious circle of nosocomial infections Of patients exposed to disseminators some 20 per cent became transient carriers and 10 per cent persistent nasopharyngeal carriers Postoperative sepsis developed in 5 per cent Out of 41 infections 13 were attributed to disseminators of enterotoxin B producing staphylococci Another 5 infections were classified as auto infections

Basically these two enterotoxin B producing strains showed a similar pattern of dissemination which contrasted markedly to that of Thorax BP This state of dissemination was in fact observed only in patient who harboured enterotoxin producing strains in their respiratory tract The cycle of colonization [illegible]nfection and dissemination occurred only in tracheotomized pati[illegible]nts r[illegible]c[illegible]iving broad spectrum antibiotics Thus [illegible]n epidemic patter[illegible] th[illegible]t [illegible]as observed in 1961 was repeated sever[illegible]l [illegible]n times 1962

No simple direct relation was [illegible]nt between environmental contamination and rate of sepsis In th[illegible]ring there was a period of heavy contamination by the 75 77 str[illegible] [illegible]though nasopharyngeal colonization was frequent frank sepsis [illegible] rare if 6 sub febrile cases with profuse sputum are excluded Th[illegible]ituation was reversed in the autu[illegible] Contamination of the environment [illegible]as not pronounced but seps[illegible] was

fairly frequent The exact cause of this discrepancy is not known It was, however, evident that the final outcome was determined by host resistance in the population at risk, degree of exposure and type of prevalent staphylococci Thus, marked exposure to strains of low infectivity led mainly to sporadic infection of infection-prone patients When high susceptibility coexisted with marked exposure to a virulent strain the result was frequently an epidemic outbreak of sepsis In contrast, marked exposure to staphylococci with known epidemic propensities led to a period of sporadic epidemic infection in a population characterized by low to moderate susceptibility Actually, sepsis seemed to occur in infection-prone patients provided that staphylococci, endogenous or exogenous, could reach susceptible loci in sufficient quantity If patients were persistent carriers at operation, they frequently contracted endogenous infection, if not, exposure to certain strains commonly resulted in exogenous infection

SUMMARY

Out of 41 postoperative staphylococcal infections 1/3 were endogenous and 2/3 exogenous Human sources—most commonly a persistent carrier or a disseminator—could be established as the ultimate origin of disease-producing staphylococci in 32 infections

Even if an exact borderline could not always be drawn between sepsis acquired in the theatre and in the wards, the intensive-care ward played a significant role in the development of 18 infections (44 per cent) It was obvious that numerous factors—such as structure of the unit, intramural movements of the patients and rate of turnover—contributed to the creation of a common source reservoir, where characteristics and prevalence of staphylococci influenced the epidemiological pattern It was nevertheless equally obvious that factors inherent in the patient and his operation were the major determinants whether a given staphylococcus in a given patient was to cause disease

Sepsis seemed to occur in infection prone patients, provided staphylococci, endogenous or exogenous, could reach susceptible loci in sufficient quantity If patients were persistent carriers at operation they frequently contracted endogenous infection, if not, exposure to certain strains commonly resulted in exogenous infection

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AN ANTIGEN FREE MEDIUM FOR CULTIVATION OF β-HEMOLYTIC STREPTOCOCCI

By

S E Holm and E Falsen

Received 10 viii 66

INTRODUCTION

Purification and isolation of antigenically and enzymatically active products from culture filtrates of β hemolytic streptococci have met with difficulties due to the complex protein nature of the media used Dialyzable media partly eliminated these difficulties and have successfully been employed by, *e g Dole* (1946) *Wannamaker* (1958) and *Halbert* (1958) Synthetic media suitable for cultivation of streptococci have also been elaborated (*e g Zampieri* 1950, *Ogburn et al* 1958, *Michelson* 1964) and would of course from a theoretical point of view be the media of choice for purification of bacterial enzymes and toxins in culture filtrates However, the yields of bacteria and bacterial products in such media are often poor

The aim of the present study was to investigate the possibility of using the gel filtration technique for preparing an inexpensive, antigen-free medium containing substances up to a certain molecular weight The growth promoting effect of the medium and the yield of some bacterial products are reported The results of some preliminary experiments to separate antigenic streptococcal products from constituents of the medium are also presented

MATERIAL AND METHODS

Yeast autolysate Commercial baker s yeast (1 kg) was thoroughly mixed with 1 liter tap water and incubated at 48-50° C for 22 hours The mixture was heated quickly to 100° C immediately cooled to room temperature neutralized with 5 N NaOH to pH 7 0 and finally centrifuged at 10 000 xg for 10 minutes The supernate (designated yeast autolysate) varied in dry weight between 5 7-6 5 per cent (w/v) The recovery was 1 3 1 5 l yeast autolysate per kg yeast In the gel filtration experi

We wish to thank *Professor Örjan Ouchterlony* for his criticism and valuable advice during the course of this work We are also greatly indebted to *Docent Bo*
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ments a twice concentrated yeast autolysate was also employed This concentration was performed on a Buchi Rotavapor R at 33° C.

Trypticase—yeast autolysate Trypticase, 27 g (BBL 02-148) was dissolved in 100 ml boiling yeast autolysate After rapid cooling 150 ml twice concentrated yeast autolysate was added and mixed thoroughly

Buffer solution In the gel filtration experiments as well as in the preparation of different growth media a buffer solution (pH 6 8) of the following composition was used 0 25 per cent $NaHCO_3$, 0 25 per cent KH_2PO_4, 0 05 per cent Na_2HPO_4, $2H_2O$

Supplement In all experiments where growth promoting effect was to be studied, the following substances were added 2 per cent glucose, 0 03 per cent cysteine HCl, 0 02 per cent l glutamine, 0 0004 per cent Ca pantothenate and 0 2

[illegible]

and eluted with the buffer solution mentioned above The elution was facilitated

[illegible]

liter

Preparation of dialyzable medium A dialyzable medium was prepared according to the description of *Halbert* (1958)

Preparation of brain heart infusion (BHI) medium Brain heart infusion, 37 g (Difco) was dissolved in 1 liter distilled water and supplemented with 2 per cent glucose and 0 03 per cent cysteine HCl Sterilization was performed by autoclaving at 120° C for 20 min

Analyses

Viscosity was measured at 20° C by means of an Oswald viscosimeter (*Kabat & Mayer* 1964)

Dry weight estimations were made after heating to 110° C for 12 hours or some what longer (up to 20 hours) on viscous samples Ash was determined after heating at 700° C for 2 hours

Qualitative amino acid analyses were performed by conventional two-dimensional paper chromatography according to *Smith* (1958) Cystine was determined as cysteic acid after *Schram et al* (1954) Tryptophan was estimated by the Fischl colorimetric method (1960) Quantitative amino acid analyses were kindly per formed by Doc Hallgren at the Sunco laboratories Goteborg Sweden according to the method of *Moore et al* (1958)

DPNase activity was determined according to the technique of *Carlsson et al* (1958)

Streptolysin O (SlO) activity was determined according to the technique of *Kalbak* (1947) and expressed in combining units (Todd units) using a standard anti streptolysin from Statens Seruminstitut Copenhagen

Streptococcal Strains

Preliminary investigations revealed [illegible]
grou[illegible]
for [illegible]
use [illegible]
str [illegible]
G were employed in [illegible]
of the new medium

[1] These strains were obtained by courtesy of *Dr Rebecca Lancefield* The Rocke feller Institute, New York.

Inoculation Cultivation and Estimation of Bacterial Growth

Inoculations were made by adding 3 per cent (v/v) of a washed streptococcal suspension (optical density 0 51) to the medium to be tested These streptococci were obtained from a BHI culture incubated at 37° C for 16 hours

Batch cultivations (in 0 8 5 liter amounts) were performed under stabilized pH conditions at pH 6 8 by means of an automatic titrator (Radiometer TTT1 Copenhagen) with 5 N NaOH as the neutralizing agent Tube cultures in triplicate were made when the growth promoting effect of different fractions was tested with strain S 84 and in the tests of the antigen free medium (TY G 25) with all 22 streptococcal strains All inoculations were performed at 37° C for 16 hours Bacterial growth was estimated as optical density (O D) in a Beckman C apparatus When necessary the cultures were diluted to give an extinction between 0 4 and 0 6 The cultures were homogenized by careful pipetting five times in the tube before the O D was measured *An optical density of 0 51 corresponded to 0 75 mg washed* and dried streptococci per ml

Antisera

Anti CF (CF = culture filtrate) serum was obtained from a sheep which received weekly injections subcutaneously for 8 weeks followed by monthly booster injections for one year Each immunizing dose consisted of 10 ml ten times concentrated culture filtrate of strain S 84 (grown in BHI medium) suspended in Freund s complete adjuvant The bleeding used was obtained after the fourth booster injection

Patient serum was obtained from a 16 year old boy who two weeks earlier had developed clinical and bacteriological signs of an acute streptococcal glomerulonephritis

Diffusion in Gel Analyses

The double diffusion in gel technique of *Ouchterlony* (1949) was employed in a modified gel chamber technique as described by *Holm* (1965) Media and culture filtrates used in the immunological analyses were concentrated on a Buchi Rotavapor R at 33° C and when necessary diluted with distilled water

EXPERIMENTS AND RESULTS

Preliminary experiments showed in agreement with *Fox* (1961) that trypsin digested casein was superior to acid hydrolysed casein for supporting growth of β hemolytic streptococci Still better growth results were obtained when yeast autolysate was incorporated in the medium *Bernheimer & Pappenheimer* (1942) and *McIlwain* (1946) reported increased growth in the presence of l glutamine and Ca pantothenate which was confirmed by our preliminary findings

Experiment 1 a Sephadex Gel Filtrations and Amino Acid Analyses

In Figs 1 and 2 representative results of gel filtrations of trypicase yeast autolysate on Sephadex G 25 and G 50 columns are shown The tubes of eluate from the Sephadex G 25 column were combined into 15 fractions each of 250 ml presence of free amino acids in the fractions was determined by paper chromatography see Table 1 The first three fractions contained only traces of free amino acids while in fractions 4 and 5 all the amino acids listed in Table 2 except phenylalanine tyrosine and tryptophan were found In fractions 6 and 7 traces of a large number of free amino acids were present but phenylalanine and tyrosine were predominating and tryphophan completely absent Tyro

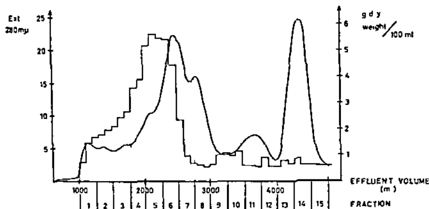


Fig 1

Trypticase—yeast autolysate separated on a 7 5 × 55 cm Sephadex G 25 column Smooth line curve represents the optical density stepped curve the ash content Fraction numbers are indicated (250 ml/fraction) All eluate in fractions 4-7 and 25 per cent of fractions 8–15 were used in preparing the TY G 25 medium

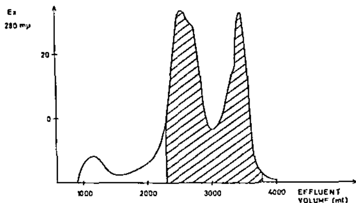


Fig 2

Trypticase-yeast autolysate separated on a 7 5 × 55 cm Sephadex G 50 column All material eluted between 2300 and 3800 ml (shaded) was used in preparing the TY G 50 medium

sine was the only amino acid found in fractions 8 and 9 No free amino acids could be detected in fractions 10 12 while in fractions 13–15 only tryphophan was recovered The molecular weight of substances eluted with fraction 1 (the void volume of the column) was above 5000 while the molecular weight of substances eluted with fractions 2 and 3 could be roughly estimated as 2500 to 5000 As may be seen in Fig 1 the ash content of the fractions largely parallelled the optical density except when amino acids known to have high molecular ab sorption like tyrosine and tryptophan were present The fractions

TABLE 1

Growth and Free Amino Acids in Fractions of Trypticase-Yeast Autolysate Separated on Sephadex G-25

	Fraction number									
	1	2 3	4	5	6	7	8	9	10–11	12–15
Free amino acids*	Trace of some		All except tyrosine, phenylalanine and tryptophan		Phenylalanine and tyrosine, traces of others		Tyrosine	Traces of tyrosine	None	Tryptophan
Growth (O D range)	0.08 0.15	0.61 0.72	0.63 0.77	0.77 0.78	0.70– 0.73	0.08– 0.10	0.05– 0.06	0.02 0.08	0 0	0 0

* All amino acids listed in Table 2

from the Sephadex G 25 column were pooled by using all material in fractions 4–7 and 25 per cent of the material in fractions 8–15 The eluates from the Sephadex G 50 column were also pooled, but in this instance all the 1500 ml of the material eluted between 2300 and 3800 ml corresponding to the shaded part of Fig 2, were combined No great differences between these two pools were found in dry weight 2 5 g/100 ml for the Sephadex G 25 pool and 2 1 g/100 ml for the G 50, ash in per cent of dry weight, 28 5 and 26 8, respectively, protein, in per cent of dry weight, 53 6 and 56 8 respectively Table 2 comprises the results of amino acid analyses of the two pools The concentrations of free amino acids were lower in the Sephadex G 25 pool than in the Sephadex G 50 The individual amino acids vary quantitatively in both instances All the amino acids described by *Slade et al* (1951) as essential for the growth of β hemolytic streptococci were found in both pools

TABLE 2
Amino Acid Analysis of the Pooled Fractions

Amino acid	Sephadex G 25 pool Free amino acids (mg/l)	Sephadex G 25 pool Free amino acids (mg/l)	Sephadex G 25 pool Total amino acids (mg/l)
Aspartic acid*	75	208	1040
Threonine	83	158	470
Serine	74	325	476
Glutamic acid	203	370	2038
Proline	48	98	771
Glycine	63	110	435
Alanine*	226	401	675
Cystine	14	39	90
Valine	190	385	764
Methionine	101	145	275
Isoleucine	168	306	618
Leucine	439	657	1142
Tyrosine	91	287	565
Phenylalanine	268	442	700
Tryptophan	25	17[illegible]	372
Lysine	361	599	950
Histidine	47	4	304
Arginine	193	208	4[illegible]4

* Amino acid not essential for growth of β hem l tic streptococci according to *Slade et al* (1951)

Experiment 1 b Growth Promoting Ef c the Fractions and Pooled Material

The growth promoting effect t lo fractions collected from the Sephadex G 25 column to wh h t aforementioned supplement had been added were studied in tr p al tuac cultures using the S 84 stra n as a test organism As may be se n in Table 1 sparse growth or none at all

was obtained in fractions 1 and 7–15, while fractions 2–6 supported good growth Supplement was added to each of the two pooled fractions (Sephadex G 25, G 50) and the media thus obtained were designated TY G 25 and TY G 50 These two media and three others (BHI medium dialyzable medium and TY medium) were employed in batch cultures using the S 84 strain as a test organism The consumption of 5 N NaOH and the optical density of these cultures (grown for 16 hours) were registered and the streptolysin O activity in the culture filtrates determined Between 3 and 10 batch cultivations were performed in each medium except the dialyzable medium where only one cultivation was made The results of these experiments are shown in Table 3 where range values with the means in italics are given It may be seen that the TY, TY G 25 and TY G 50 media promoted a heavy growth of the test strain and that a large quantity of 5 N NaOH was consumed to maintain the pH at a constant level (6 8) during the growth period The dialyzable medium also promoted heavy growth and the consumption of 5 N NaOH was high The growth in the BHI medium was good but the highest O D value noted was less than the lowest value for the TY, TY G 25 and TY G 50 media Comparable results were obtained for the consumption of 5 N NaOH

In Table 3 it may also be seen that the streptolysin O activity varied in the culture filtrates from the different media The mean activity for the BHI batches was only half the mean levels for the gel filtered media In some of these batches the culture filtrates were tested for DPNase activity The activity was found to be as high as 1800–1900 units/ml culture filtrate when the TY G 25 or TY G 50 media were employed as compared to 800 1000 units/ml when the BHI medium was utilized

TABLE 3

Growth Response and Streptolysin O Production of Strain S 84 Cultivated in Various Media Mean Values in Italics

Medium	No of batches	Bacterial growth (in O D)	Consumption of 5N NaOH ml/l culture	Streptolysin O activity (units/ml culture filtrate)
Brain heart infusion	10	0 5–*0 7* 0 9	11 *17* 29	8–*12* 16
Dialyzable	1	2 1	31	16
TY	3	2 5–*2 6* 2 7	35 *37* 40	16–*32* 40
TY G 25	10	1 9 *2 1* 2 4	35 *37* 39	16 *24*–27
TY G 50	5	2 2 *2 5* 2 8	37 *40* 41	16 *24* 40

Experiment 2 TY G 25 as a Streptococcal Growth Medium

Ten different strains of β hemolytic streptococci belonging to the Lancefield groups A, C and G were cultivated in the TY G 25 medium

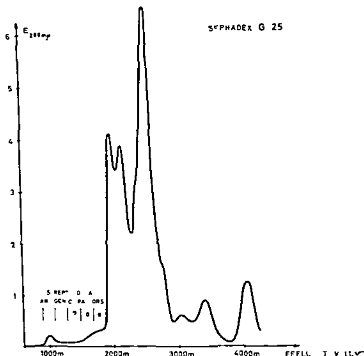


Fig 3

The figure shows the elution curve for a culture filtrate from streptococci grown in TY G 25 medium separated on a 7 5 × 55 cm Sephadex G 25 column All precipitinogenic streptococcal material was eluted with the first small protein peak.

under the aforementioned stabilized pH conditions The optical density of these cultures varied between 2 1 and 3 9 (mean 2 9) 31–43 ml (mean 38 ml of 5 N NaOH were consumed per liter culture For purposes of comparison the same strains were also cultivated in BHI medium under analogous experimental conditions The bacterial growth in these cultures varied between 0 3 and 1 2 (mean 0 6) and the amount of 5 N NaOH required to maintain the pH at a constant level was 9 5–25 ml (mean 18 ml) per liter culture

The growth promoting effect of the TY G 25 medium was also tested in tube cultures without pH adjustment using 22 different strains of β hemolytic streptococci belonging to the serologic groups A C and G Abundant growth (O D > 1 0) was registered in all cultures after 16 hours' incubation at 37° C

Experiment 3 Purification of Strept cal Antigens from Cultures Grown in TY G 25 Medi m

To investigate the possibi ty of u r g gel filtration for isolation of precipitating streptococcal antig ns th following experiment was p r formed One liter of a culture filtrat from strain S 84 grown under

stabilized pH conditions in TY G 25 medium was concentrated 20 times in a Buchi Rotavapor This concentrated material was applied to a 7 5 × 55 cm Sephadex G 25 column The fractionation was performed as described before but the elution was made with a 0 05 M phosphate buffer (pH 7 0) In Fig 3 the results of this fractionation experiment are illustrated The first 800 ml eluted were discarded while the effluent between *800 1800 ml* was divided into 50 ml fractions and each con centrated 10 times The antigenic content of these fractions was com pared with that of the 20 times concentrated culture filtrate by means of the double diffusion in-gel method employing anti CF serum and the patient serum As indicated in the figure the precipitating streptococcal antigens were found in the first small peak eluted from the column well in advance of the major constituents of the medium The 16 precipitating streptococcal factors demonstrated in the original con centrated culture filtrate were recovered in the early 50 ml fractions Streptolysin O as well as DPNase activity were also found in the same fractions

Another liter aliquot of the same culture filtrate was precipitated with ammonium sulfate at 85 per cent saturation at 4° C The pre- cipitate was dissolved in 10 ml 0 05 M phosphate buffer (pH 7 0) and dialyzed against the same buffer at 4° C for three days final volume 12 5 ml This highly concentrated (80 times) preparation contained 20 precipitinogens when analyzed with the double diffusion in gel tech nique against the anti CF serum or the patient serum

Experiment 4 Antigenicity Testing of TY G 25 and TY G 50

In order to test the media for possible antigenicity 6 rabbits (two rabbits for each medium) were injected subcutaneously with ten times concentrated TY TY G 25 and TY G 50 media The immunization schedule employed was the same as that used for the preparation of anti CF in sheep Each injection consisted of 2 ml concentrated medium in complete Freund s adjuvant Bleedings were performed after the fourth booster injection and the hyperimmune sera designated anti TY anti TY G 25 and anti TY G 50 These antisera and the anti CF pro duced by immunization with filtrates from BHI cultures were analyzed *with the double diffusion in gel method against the different media*

As indicated in Table 4 five precipitation lines were observed when the TY medium was analyzed against anti TY serum but no lines were seen when the same medium was tested against the other antisera When the TY G 25 and TY G 50 media were tested against the hom logous and heterologous immune sera no precipitation lines were ob served while the dialyzable medium reacted with anti TY and anti CF by forming one line with each of these sera The BHI medium only reacted with anti CF at least four distinct precipitates were formed In comparative analyses it was found that these four lines coalesced

with four of the sixteen lines produced when a 20 times concentrated filtrate from a streptococcal culture grown in BHI medium was analyzed against anti CF

TABLE 4

Number of Immunoprecipitates Noted at Diffusion in Gel Analysis of each Medium with the Various Hyperimmune Sera

Medium	Antiserum			
	anti TY	anti TY G 25	anti TY G 50	anti CF
TY	5	0	0	0
TY G 25	0	0	0	0
TY G 50	0	0	0	0
Dialyzable	1	0	0	1
BHI	0	0	0	4

DISCUSSION

One of the first media specifically elaborated for growth of β hemolytic streptococci was presented by *Todd & Hewitt* as early as 1932 The factors essential for cultivation of β hemolytic streptococci have since been studied by many authors employing partially defined semisynthetic or synthetic media (e g *McIlwain* 1940, *Bernheimer & Pappenheimer* 1942, *Adams & Rose* 1945 *McIlwain* 1946 *Slade & Knox* 1950 *Zampieri et al* 1950, *Slade et al* 1951, *Ogburn et al* 1958 *Fox* 1961, *Michelson* 1964) From the results of these investigations it could be concluded that a suitable inexpensive base medium for cultivation of streptococci could be produced by employing trypsin digested casein as the essential source of amino acids and peptides and, in addition, yeast extract as the source of purines pyrimidines and vitamins of the B complex Furthermore the presence of a variety of trace elements in yeast extracts (*Grant & Pramer* 1962) may have an additional growth stimulating significance Various types of supplements have also been employed in streptococcal media to implement the growth promoting effect or the streptolysin O production The composition of the supplement used in the present study was based on the results reported by *Bernheimer & Pappenheimer* (1942) and *Slade & Knox* (1950)

The Sephadex gels are known to separate most substances according to molecular weight and to permit heavy loads (*Gelotte* 1960, *Flodin* 1962, *Laurent & Killander* 1964) Filtration with Sephadex G 25 or G 50 would then represent a convenient way of preparing a growth medium containing substances of a low molecular size and would avoid laborious and time consuming processes like dialysis salt precipitation or elaboration of synthetic media The results of the filtration experiments showed that a good separation of high molecular material(eluted with the void volume) from low molecular substances was achieved Only traces of free amino acids were eluted with the first three fractions

from the Sephadex G-25 column charged with trypticase—yeast autolysate The analyses of the pooled fractions used in preparations of the TY G 25 and TY G-50 media showed that all amino acids, stated by *Slade et al* (1951) to be essential for cultivation of streptococci, were present in free form The quantities of these amino acids were small compared to the amounts recommended by these authors but essentially higher than the amounts which, according to *Snell* (1951), allow maximal growth of fastidious lactic acid bacteria Furthermore, *Slade et al* (1951) discussed the possibility that streptococci can utilize some peptides directly for the synthesis of their own proteins, the large amount of low molecular peptides in the gel-filtered media might compensate for a low concentration of free amino acids

The results of the cultivation of streptococcal strain S 84 (Group A, type 3) in the TY G 25 and TY G 50 media and the unfiltered TY medium showed that a heavy growth was achieved in all three media Only minor differences could be noticed concerning consumption of 5 N NaOH, bacterial growth and streptolysin O production Hence the high molecular weight substances in the TY medium had no specific growth stimulating effect The brain heart infusion medium, which was used as a commercially available reference medium for streptococci, did not promote as good growth as the gel filtered TY media This difference was especially pronounced when 10 different streptococcal strains from groups A, C and G were cultivated under identical conditions In these experiments more than twice as much 5 N NaOH (on the average) was consumed and the bacterial growth was more than three times as dense (on the average) in cultures grown in TY G-25 media compared to cultures grown in BHI medium That the good growth promoting effect of the TY G-25 medium was not an accidental occurrence for certain streptococcal strains was further verified by tube cultivation employing 22 different strains of β-hemolytic streptococci belonging to the Lancefield's groups A, C and G All the strains showed an abundant growth (O D > 1.0) after 16 hours' cultivation in the TY G-25 medium

Purification of bacterial antigens in culture filtrates has become a problem of specific interest since it was found that the streptococcal enzyme streptokinase may be utilized in the treatment of medical disease, *e g* thrombosis In the present investigation a purification experiment was performed by submitting concentrated culture filtrates of streptococci grown in TY G-25 medium to filtration on Sephadex G-25 columns The streptococcal antigenic factors were eluted with the void volume, *i e* separated from the low molecular constituents of the TY G 25 medium in a single step It was also shown that ammonium sulfate precipitation (at 85 per cent saturation) was a convenient way to concentrate streptococcal antigens in filtrates from cultures grown in TY G-25 medium When such a streptococcal antigen preparation was analyzed in double diffusion-in-gel plates against a hyperimmune serum from a sheep (anti CF) or a serum from a patient with streptococcal

glomerulonephritis more than twenty precipitation bands were seen Similar results have been reported by *Halbert* (1959) and clearly illustrate the multiplicity of antigens in culture filtrates from streptococci

In the immunological experiments performed to study the antigenicity of the gel filtered media no antigen-antibody reaction could be demonstrated between the media and their homologous antisera The lack of antigenicity in the TY G 25 and TY G 50 media was further illustrated by analyzing these media against anti-TY sera Although the TY medium stimulated antibody production in the rabbits these antibodies did not react with the TY G-25 or the TY G 50 media The dialyzable medium employed was not antigen-free as demonstrated by diffusion-in-gel analyses with both anti-TY and anti CF. Also the BHI medium was found to contain precipitinogenic factors when this medium was analyzed against anti CF serum One of these factors was common with a factor in the dialyzable medium

Both the TY G–25 and TY G–50 media seem to fulfil the criteria for an all round streptococcal medium since they are antigen-free, promote good and rapid growth, stimulate the production of extracellular streptococcal antigens and are suitable for isolating high molecular weight streptococcal products from constituents of the medium It is difficult to choose between these media, but the higher flow rate through Sephadex G 25 compared to Sephadex G 50 dictated the use of TY G-25 in the present study These results are largely in accord with preliminary results on this topic reported by *Falsen & Holm* (1964)

SUMMARY

The preparation of streptococcal media containing elements of low molecular size by gel filtration of trypticase-yeast autolysate through Sephadex G-25 and G 50 is described Amino acid analyses of the media showed that all amino acids known to be essential for the growth of β-hemolytic streptococci were present in a free form Heavy growth and high streptolysin O activity were produced by β hemolytic streptococci in these media and at higher levels than in a commercially available streptococcal medium Immunological experiments showed that the gel filtered media lacked antigenic properties and were suitable for the preparation of extracellular streptococcal antigenic materials freed from medium constituents

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Acta path et microbiol scandinav 69 277-286 1967

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AN INTRACELLULAR INHIBITOR OF STREPTOCOCCAL DPNase IN BETA-HEMOLYTIC STREPTOCOCCI

By

S F Holm and B Kaijser

Received 10 viii 66

Since *Carlson et al* (1957) first described a streptococcal DPNase[1] acting on the nicotinamide-ribose linkage of DPN the enzyme has been studied in different aspects (*Bernheimer et al* 1957, *Lazarides & Bernheimer* 1957, *Kellner et al* 1958, *Bernheimer* 1960, *Petersen* 1961, *Petersen et al* 1961 *Petersen* 1962a, 1962b, *Holm & Kaijser* 1965) Its role in streptococcal infections is not clear *Bernheimer et al* (1957) suggested an association between DPNase production and leucotoxic activity DPNase has also been claimed to influence the development of streptococcal nephritis (*Bernheimer* 1960) *Kellner et al* (1958) and *Petersen* (1962) observed raised DPNase antibody levels after streptococcal infection in man

The aim of the present investigation was to study the production of DPNase by streptococci and the protection of the microorganism against the enzyme The DPNase activity in extra and intracellular preparations from streptococcal strains belonging to Lancefield's groups A, C and G was investigated The final results of a preliminary report (*Holm & Kaijser* 1965) on the characteristics of an intracellular DPNase inhibitor in certain streptococcal strains are given

MATERIAL AND METHODS

Streptococcal strains Ten beta hemolytic streptococcal strains[2] from Lancefield's groups A C and G were investigated The strains used are listed in Table I and the Lancefield designations are given

Preparation of extracellular material (EC) The streptococci were grown in brain heart infusion broth (Difco 37 g/l) supplemented with sterile filtered cysteine HCl

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[1] DPN (Disphosphopyridine nucle[illegible] [illegible] wa[illegible] always often called NAD Nicotinamidadeninedinucleotide

[2] These strains were kindly suppl[illegible]d by *Dr R[illegible]becca Lancefield* The Rockefeller Institute New York

We wish to express our deep gra[illegible]ide t[illegible] *Pr*[illegible] valuable criticism We are also [illegible] and Eng *Enevold Falsen* *Mrs* [illegible] script The skilful technical ass[illegible]an[illegible] of *M*[illegible] *Wikström* is very much appreciated

and glucose at final concentrations of 0 03 per cent and 2 per cent respectively In parallel experiments the gel filtered antigen free medium described by *Falsen & Holm* (1964) was employed The cultivations were performed under stabilized pH conditions at 6 2 and 6 8 using an automatic titrator and employing 5 N NaOH as the neutralizing agent Estimation of growth was performed by measuring the optical density as described in a preceding paper (*Falsen & Holm* 1964) After sixteen hours of cultivation at 37° C the streptococcal cultures were centrifuged at 1500 xg for half an hour at 4° C The supernatant was filtered through a Göttingen No 6 membrane at 4° C and kept in the cold room This material was designated extracellular material (EC)

Preparation of intracellular material (IC) The wet bacterial sediment from each culture was washed three times with saline and finally suspended in 4 volumes of buffered saline pH 7 0 (M/15 phosphate buffer) The suspension was homogenized in a Raytheon 200 watt 10 kc oscillator for half an hour and thereafter freezepressed five times in the X press described by *Edebo* (1960) After thawing the pressed material was mixed with glass beads and shaken at 4° C for three hours and finally centrifuged at 12000 xg at 4° C for half an hour The clear slightly yellow supernate was designated IC (intercellular material)

Anti EC serum was obtained from a sheep which had been immunized by repeated subcutaneous injections of EC from strain S 84 (Group A Type 3) using Freund s adjuvant technique The immunization schedule implied weekly injections of 10 ml ten times concentrated culture filtrate during 8 weeks followed by one monthly injection of the same amount for one year Bleedings were performed one week after the last injection

Anti IC + EC serum was obtained from a sheep immunized in a similar way as described above by injections of mixtures of IC and EC of several strains of group A streptococci

Anti IC sera were produced with Freund s adjuvant technique in rabbits by six weekly injections of IC from one DPNase negative (T1 119 7) two DPNase positive group A strains (S 84 and B 737 34 41) and from one moderately DPNase positive group G strain (F 68 A) The corresponding immune sera were designated anti IC A1 anti IC A3 anti IC A49 and anti IC G

Anti IC A3 + IC A1 Equal amounts of anti IC A3 and IC A1 were incubated at 37° C for 30 min and stored overnight at 4° C The supernatant after centrifugation at 1500 xg for 20 min was decanted and designated anti IC A3 + IC A1

Trypsin[1] treatment of IC One ml trypsin solution (1 mg/ml M/15 phosphate buffer pH 8 0) was added per ml IC and the mixture was incubated for 30 minutes at 37° C Trypsin inactivation was achieved by adding 1 ml of a buffered saline solution pH 8 0 of soy bean trypsin inhibitor (1 mg/ml NBC England) The final DPNase inhibitory capacity was tested by means of a preparation with known DPNase activity (800 units ml) The effect of the various reactants were tested as shown in Table 2

Gel filtration of IC IC material was fractionated on Sephadex G 75 columns (2 5 × 82 cm) using a 0 05 M phosphate buffer pH 7 0 in 0 5 M NaCl The fractions were collected by means of a LKB fraction collector using a 5 ml siphon and concentrated in visking tubing (kebo T 106) against polyethylene glycol 6000 As reference substances with known molecular weight recrystallized preparations of lysozyme and/or pepsin (NBC England) were filtered through the same Sephadex column

Dialysis of IC Dialysis was performed in visking tubing (kebo T 106) with a pore diameter of 50 Å

Ultrafiltration of IC Ultrafiltrations were performed on the LKB model 6300 A ultra filter at 4° C According to the manufacturer s specification the pore diameter corresponds to a molecular size of about 30000

DPNase determination was made according to the technique of *Carlson et al* (1957) This is a slight modification of the method described by *Kaplan et al* (1951) one unit being the amount of enzyme that destroys 0 01 µM DPN in 7 5 minutes at 37° C In our tests the DPN concentration after addition of NaCN in excess corresponded to an extinction of 0 6 in a Beckman DB spectrophotometer (340 mµ 1 cm cuvettes) The decrease in optical density was linearly related to DPNase activity only in a limited region (ext 0 45 0 20

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1 Trypsin 1 200 Difco certified

DPNase inhibition test In these tests we have chosen a DPNase concentration corresponding an extinction of 0 25 Thus higher extinction values correspond to the presence of DPNase inactivating substances

IC preparations (0 05 ml) and the same amount of the DPNase solution (in 0 1 M phosphate buffer pH 7 3) were mixed After 2 min at room temperature 0 4 ml of the DPN solution mentioned above was added and the mixture was incubated for 7 5 min at 37° C The enzymatic action was stopped by cooling in ice water and the addition of 3 ml N NaCN The optical density was measured Suitable blancs and controls were run in parallel DPNase inactivation by anti DPNase and inhibitor neutralizing effect of anti inhibitor sera were analysed in a similar way in all sera used

The quantitation of DPNase inhibition was based on the DPNase unit— one inhibiting unit being the amount that inhibits one unit of DPNase

Immunological analyses Comparative double diffusion in gel analyses were performed according to the method of *Ouchterlony* (1958) in a modification described by Holm (1965) In the immunoelectrophoretic analyses the technique described by *Wadsworth & Hanson* (1960) was used an elaboration of the original method of *Grabar & Williams* (1953) Adequate separations were obtained on 9 × 12 cm glass plates with a field strength of 7 V per cm applied for 80 minutes The antigen basins were placed near the cathode as most of the precipitinogens in IC move towards the anode during the electrophoresis Photographic registration of the precipitation patterns was performed according to the description of *Wadsworth* (1963)

TABLE 1

Intracellular DPNase Inhibitor Effect Compared with Intra and Extracellular DPNase Activity in Various Strains of Streptococci

Beta streptococcus Group	Type	Strain*	DPNase activity in EC[1]	DPNase activity in IC[2]	DPNase inhibition in IC[2]
A	1	T1 119 7	—	—	—
A	3	S84	+++	—	+++
A	3	C 203S	+++	—	+++
A	4	T4 95 Rb5	+++	—	++
A	49	B737 34 41	+++	—	+++
A	50	B514-33 3	+++	—	++
C		K64 0 13	—		—
C	20	T20J(Neil)	++		++
G	16	T16 J	++		++
G		F68 A	++	—	+

— [illegible]
+ [illegible]
++ [illegible]
+++ [illegible]
[1] EC — Extracellular material
[2] IC — Intracellular material
* The strain numbers refer to the designation made by R Lancefield

RESULTS

DPNase Activity in Extra- and In[illegible]*lular Materials*

As may be seen in Table 1 DPN[illegible]tivity was found in EC from 5 of the 6 group A strains tested DPNase activity was not demonstrated in the sixth strain (T 1 119[illegible] highest DPNase activity (1800 units/ml) was detected in cultu[illegible]ltrates from the two Type 3 strains when cultivated in the antigen fr[illegible] medium One of the two group C

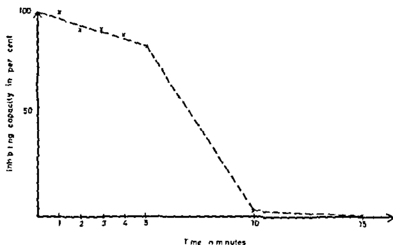


Fig 1

Figure showing the inactivation of the intracellular DPNase inhibitor in relation to time at 60° C The inhibitor power at different intervals is expressed as per cent of the initial inhibitory capacity of intracellular material

strains produced moderate amounts of DPNase while the other was DPNase negative The two group G strains tested showed moderate DPNase activity No significant differences in DPNase production were noted if the cultivations of the different strains were performed at pH 6 8 or 6 2

In no experiment was active DPNase found in IC from any of the strains tested

DPNase Inhibition by Intracellular Materials

Table 1 also shows the inhibitory capacity of IC from streptococcal strains belonging to groups A, C and G and the DPNase activity in the corresponding culture filtrates The highest inhibitory capacity was found in IC from the two Type 3 strains which also showed the greatest extracellular DPNase activity Furthermore it was found that the inhibitor was active not only against DPNase from the corresponding strain or type but also against DPNase produced by streptococci belonging to other serological groups As may be seen only DPNase producing strains showed DPNase inhibiting effect in IC

Characterization of the DPNase Inhibitor

Thermostability Storage for 6 months at 4° C did not inactivate the DPNase inhibitor in IC Nor did heat treatment for 45 min at 37° C lower the inhibitory effect However, at 60° C a complete inactivation of the DPNase inhibitor was obtained in 15 min (Fig 1)

Heat treatment of EC for 10 min at 60° C completely destroyed the DPNase activity

Trypsin stability The results of trypsin treatment of the inhibitor are shown in Table 2 As may be seen the DPNase inhibitor was destroyed by trypsin It was also shown that neither DPNase nor the inhibitor were affected by the soy bean trypsin inhibitor used to inactivate the trypsin

TABLE 2
Effect of Trypsin on DPNase Inhibitor

	Proportion of reactants				
DPNase *inhibitor*	Trypsin *solution*	Soy bean trypsin *inhibitor* solution	Buffer *solution*	DPNase units added	Final DPNase activity (in units)
0	0	0	3	800	800
1	0	0	2	800	< 100
1	1	1	0	800	800
1	0	1	1	800	< 100
0	1	1	1	800	800
0	0	1	2	800	800

Dialysis and ultrafiltration Dialysis of IC in Visking tubing with a pore diameter of 50 A against buffered saline showed that the inhibitor was not dialysable Ultrafiltration experiments showed, however, that the inhibitor appeared to be at the borderline of penetrability through the ultrafilter permitting passage of substances of a molecular size up to 30 000

Gel filtration Fractionation experiments of IC were performed on Sephadex G 75 columns In Fig 2 a fractionation experiment of IC A3 (S 84) is illustrated High DPNase inhibitory activity was found in

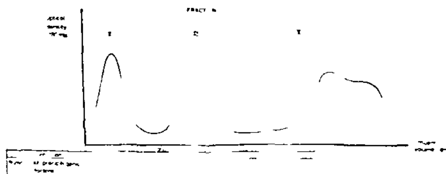


Fig 2

A curve represe nts separati on of intracellular material (S 84) on a Sephadex G 75 column Th DPNase inhibitory activ ty was almost exclusively found in fraction III while most of the other intracellular precipitinogenic factors were eluted in fractions I and II

fraction III Fractionation of IC from five other DPNase positive strains showed almost identical results No inhibitory effect could be detected in any fraction of separated IC from DPNase negative strains In order to evaluate the molecular size of the inhibitor, reference substances with a molecular weight of 17,500 and 34,500 (lysozyme and pepsin respectively) were filtered through the same Sephadex column These two substances were eluted on either side of the protein peak of IC fraction III which indicated that the molecular size of the inhibitor could be roughly estimated at 20,000–30,000

Antigenicity Sera from rabbits immunized with IC preparations from DPNase positive and negative strains were analysed for the presence of inhibitor neutralizing antibodies The results of the analyses of two anti-sera are summarized in Table 3 As may be seen the DPNase inhibitory effect of IC A3 (S84) was neutralized by the corresponding antiserum This antiserum also neutralized the inhibitory effect of IC from the other type 3 strain tested It is further evident from the table that the inhibitor neutralizing antibodies were not type or group specific as the antibodies in anti-IC A3 could also neutralize the inhibitory effect in IC from streptococci belonging to other group A strains as well as to groups C and G Anti-IC A3 (S84) did not inactivate DPNase in EC from any of the DPNase positive strains tested Antiserum produced with a DPNase negative strain (anti-IC A1) did not neutralize the inhibitory effect of IC preparations from any DPNase positive strain, nor were DPNase antibodies detected in this serum

TABLE 3
Inhibitor Neutralizing Effect of Anti IC A3 and Anti IC A1 on IC Preparations from certain Streptococcal Strains

Beta streptococcus			Inhibitor neutralizing effect of	
Group	Type	Strain	Anti IC A3	Anti IC A1
A	3	S84	+	—
A	3	C203S	+	—
A	4	T4 95 Rb5	+	—
A	49	B737 34 41	+	—
A	50	B514 33 3	+	
G	20	T20J (Neil)	+	
G	16	T16J	+	—

The immunoelectrophoretic pattern obtained when IC A3 (S84) was separated and analysed against anti-IC + EC serum is illustrated in Fig 3 At least twenty separate precipitation lines were revealed with the crude IC, but when Sephadex gel fraction III of IC A3 (S84) was tested against anti-IC + EC serum only two precipitation lines were found These lines were designated xI and xX Only the xX immunoprecipitation band was observed when fraction III of IC A1 (lacking DPNase inhibiting effect) was analysed in the same way against anti IC + EC and

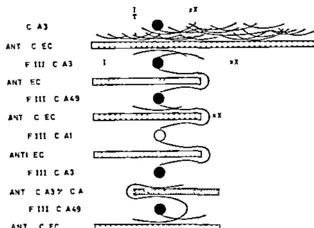


Fig 3

Schematic drawing of immunoelectrophoretic separations of streptococcal intra cellular material or fractions thereof developed with different anti streptococcal sera *Filled circular basins* DPNase inhibitory materials *Open circular basin* Material lacking DPNase inhibitor *Dotted long basins* Immune serum containing DPNase inhibitor neutralizing antibod es *Shaded long bas ns* Immune serum lacking DPNase inhibitor The inhil itor—anti inhibitor immune precip tate s designated II

anti EC serum This precipitation line (xX) was also the only one seen when fraction III of IC A3 (S84) was analysed against anti EC serum which lacked inhibitor neutralizing antibodies

The precipitation pattern in the lower part of the figure illustrates the antigenic relationship between the *x* factors in sephadex gel fractions III of IC from two different inhibitory positive group A streptococcal strains analysed against anti IC A3 (S84) serum which had previously been absorbed with IC A1 Only one precipitinogen was revealed in each fraction and the coalescence of the lines indicated the immunological identity of the *x* antigen in the two fractions Serological reactions of identity shows that the *x* antigen was common to IC and EC of DPNase positive and negative strains Similar results were obtained using the comparative double diffusion technique of Ouchterlony

DISCUSSION

Since DPN is an importance coenzyme in cellular respiration it is likely that the bacterial cell producing DPNase must also have the capacity to control the activit f the latter Such a control mechanism has been described by *Bernheimer et al* 1951) who observed that streptococcal RNA inhibits action of st ptococcal DNAse

In our studies most hu an pathogenic s reptococcal strains belon ing to Lancefield s group A C and G w capable of producing DPNase which was detected in I C The on DPNase negative stre t oc i

fraction III Fractionation of IC from five other DPNase positive strains showed almost identical results No inhibitory effect could be detected in any fraction of separated IC from DPNase negative strains In order to evaluate the molecular size of the inhibitor, reference substances with a molecular weight of 17,500 and 34 500 (lysozyme and pepsin respectively) were filtered through the same Sephadex column These two substances were eluted on either side of the protein peak of IC fraction III which indicated that the molecular size of the inhibitor could be roughly estimated at 20,000–30,000

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A	3	S84	+	—
A	3	C203S	+	—
A	4	T4 95 Rb5	+	—
A	49	B737 34 41	+	—
A	50	B514 33-3	+	—
G	20	T20J (Neil)	+	—
G	16	T16J	+	—

The immunoelectrophoretic pattern obtained when IC A3 (S84) was separated and analysed against anti IC+EC serum is illustrated in Fig 3 At least twenty separate precipitation lines were revealed with the crude IC but when Sephadex gel fraction III of IC A3 (S84) was tested against anti IC+EC serum only two precipitation lines were found These lines were designated ιI and κλ Only the κλ immunoprecipitation band was observed when fraction III of IC A1 (lacking DPNase inhibiting effect) was analysed in the same way against anti-IC+EC and

that an intracellular inhibitor may be liberated during long term cultivation resulting in depressed DPNase activity

Schwarz et al (1956) have described a DPNase inhibitor and its corresponding enzyme produced by Proteus vulgaris and Bacillus subtilis However, these DPNase attack the pyrophosphate group of DPN and not the linkage between nicotinamide and ribose as the streptococcal DPNase does

Kern (1956) detected a DPNase and a corresponding inhibitor in *Mycobacterium butyricum* This enzyme cleaves the nicotinamide—ribose linkage of DPN just as the streptococcal DPNase does In bacterial extracts he found no DPNase activity because of the presence of an active inhibitor By boiling he could selectively destroy the inhibitor and thus produce an enzymatically active intracellular extract In the present study intracellular materials from DPNase positive streptococci were also found to lack DPNase activity However, heat treatment could not be used to selectively inactivate the inhibitor because streptococcal DPNase was as heat sensitive as the inhibitor

SUMMARY

Intracellular preparations from DPNase positive streptococcal strains were found to contain an antigenically active DPNase inhibitor The results of immunological analyses showed that the inhibitor was neither type nor group specific A protein nature was suggested by the results of heat and trypsin treatment The molecular weight could be roughly estimated at 20000–30000

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Acta path et microbiol scandinav 69 287-303 1967

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RESPIRATORY VIRUS DISEASE IN INFANCY AND CHILDHOOD IN COPENHAGEN 1963–65

An Estimation of the Aetiology Based on Complement Fixation Tests

By

ALLAN HORNSLETH

Received 31.x 66

A substantial part of the respiratory infections in infancy and childhood can now be associated with several agents which are known to be responsible for different proportions of the various respiratory disease syndromes Even though the complement fixation test has been shown to be of comparatively low sensitivity in these age groups (3 29 42) it is still the most convenient test for use in epidemiological surveys Reports from recent years (15 31 44) have clearly established that the respiratory syncytial (RS) virus and the parainfluenza viruses are more important as aetiological agents for both upper and lower respiratory tract disease in childhood than the influenza viruses while the adeno viruses take up a position intermediate between these

Paired sera from infants and children admitted to hospitals in Copenhagen over the years 1963 1964 and 1965 mainly for respiratory tract disease have been examined for complement fixing antibodies against influenza parainfluenza adeno and respiratory syncytial viruses It has been attempted to establish the frequencies of infections caused by the viruses mentioned which can be diagnozed by means of complement fixation tests It has been shown that virus isolation is a more sensitive method for detecting infection than the complement fixation test in parainfluenza adeno and respiratory syncytial virus infections in infancy (3 30 42 4 29) The moderate sensitivity of the complement fixation tests employed was taken into consideration when the results presented in this paper were evaluated

MATERIAL AND METHODS

The material cons[illegible] f pa[illegible]red sera [illegible] from pa[illegible]diatric departments in hospitals in Copenhagen during the pe[illegible] 1963 t[illegible] May 1965 Only sera from the age groups 0-9 years have been examined

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The auth[illegible] [illegible] to D[illegible] *P[illegible]n Magnus* M D Statens Serum[illegible]nst[illegible]tut Copenhagen f[illegible] m[illegible]ssion to [illegible] [illegible] tests for complement fixing an[illegible]bod[illegible]es to aden[illegible] and influenza virus performed in the Influenza departmen[illegible] at Statens Serum[illegible]

In the Influenza department at Statens Seruminstitut 473 paired sera have been tested for complement fixing antibodies against influenza A B C and adeno virus Of these 473 sera we have tested 404 for antibodies against the RS virus 398 for antibodies against parainfluenza virus type I 329 for antibodies against para influenza type II and 368 for antibodies against parainfluenza virus type III Furthermore 272 of the above mentioned 473 sera have been tested for complement fixing antibodies against ornithosis

Antigens employed 1) RS virus complement fixing antigen prepared in Hep 2 cell cultures as described previously (18) 2) Parainfluenza I viral antigen (C F) Burroughs Wellcome & Co London 3) Parainfluenza II (CA) C-F antigen Micro biological Associates Bethesda U S A 4) Parainfluenza III (HA 1) C-F antigen Microbiological Associates Bethesda U S A 5) Ornithosis phosphatide antigen pre pared as described previously (45) 6) Mumps antigen Behringswerke Marburg Germany 7) Influenza A B and C complement fixing antigens were prepared in the Influenza department in embryonated eggs employing the following virus strains A I R8 B Lee and C Am 1233 8) Adenovirus complement fixing antigen was pre pared in the influenza department from HeLa cell cultures infected with the adeno virus type 4

Complement fixation tests employed 1) For RS virus antigen as earlier described (17) 2) For all the parainfluenza virus antigens and with the ornithosis antigen and the mumps antigen mentioned above the technique of *Bradstreet & Taylor* (1) has been used 3) In the Influenza department at Statens Seruminstitut the tech nique of *Fulton & Dumbell* (12) as modified by *Svedmyr* et al (37) was employed in tests for influenza and adeno virus complement fixing antibodies

Control antigens and both positive and negative control sera were included in all tests series

Intervals between the collection of the paired sera were from 5 to 18 days with a mean of 10 days All sera were heated for 30 minutes at 56° C and stored at —20° C until examined

Abbreviations used 1) C-F = Complement Fixation 2) P I = Parainfluenza type I 3) P II = Parainfluenza type II 4) P III = Parainfluenza type III 5) RS = Respiratory Syncytial 6) Infl C = Influenza type C

EXPERIMENTAL

Occurrence of Antibodies against Parainfluenza Type I Complement Fixing Antigen

Fig 1 shows the distribution according to age of 398 paired sera examined with this antigen It can be seen that the majority of sera comes from patients below the age of three years Fig 1 also shows the age distribution of sera containing antibodies (dilution 1 8 being the lowest dilution examined) and of sera showing more than a two fold increase in titre The percentage of sera showing presence of anti bodies rises from 11 per cent in the age group 0 years to 63 per cent in the age group 4 years There is only a slight increase in the percentage of sera showing presence of antibodies in the age groups above 4 years This fact is illustrated in Fig 4 It can be seen from Fig 1 that there is no accumulation of sera showing more than a two fold increase of titre in any particular age group

Occurrence of Antibodies against Parainfluenza Type II Complement Fixing Antigen

Fig 2 presents the distribution according to age of 329 paired sera examined with this antigen and of sera containing antibodies (indicated

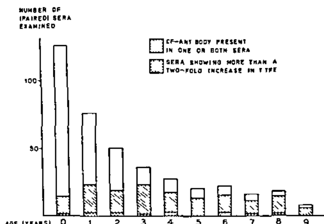


Fig 1

Distribution according to age of 398 paired sera from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965
All sera were examined for C-F antibodies to parainfluenza virus type I
Those showing presence of antibody are indicated

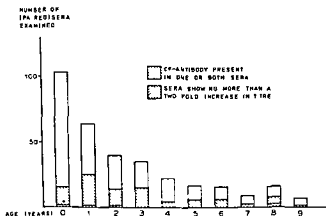


Fig 2

Disti

in the same way as in Fig 1) The percentage of sera showing presence of antibodies (dilution 1 8 being the lowest dilution examined) rises from 15 per
3 years In t
bodies show
being due to the small number of sera examined in the older age groups

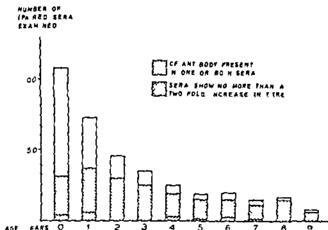


Fig 3

Distribution according to age of 368 paired sera from patients admitted to hospitals in Copenhagen during the period Septeml er 19f3 to May 1965
All sera were examined for C-F antibodies to parainfluenza virus type III
Those showing presence of antibody are indicated

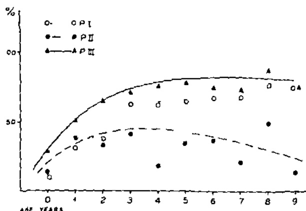


Fig 4

Distribution according to age of percentages of paired sera containing C F antibodies to parainfluenza viruses type I II and II from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965

Because of the difference in numbers of sera examined in the different age groups it is not possible to say whether there is accumulation of sera showing more than two fold increase of titre in any age group

Occurrence of Antibodies against Parainfluenza Type III Complement Fixing Antigen

Fig 3 shows the distribution according to age of 368 paired sera examined and of sera found to contain antibodies against this antigen (serum dilution 1 8 being the lowest dilution examined) The per-

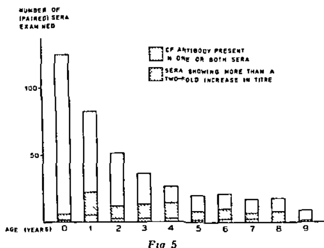


Fig 5

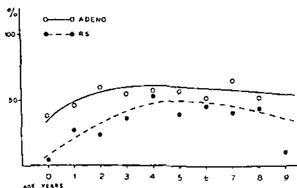


Fig 6

Distribution according to age of percentages of paired sera containing C–F antibodies to adeno and RS viruses from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965

centage of sera containing antibodies rises from 29 per cent in patients below 1 year to approximately 80 per cent in the age groups above 5 years There is no definite accumulation of paired sera showing more than a two-fold increase of titre in any age group examined with this antigen

In Fig 4 the percentage of sera containing antibodies against the three parainfluenza C–F antigens mentioned above in the age groups 0–9 years are shown It can be seen that the percentage of sera containing antibodies against type III antigen increases more steeply and reaches a higher level than the percentages of sera containing antibodies

against type I and II antigens Fig 4 demonstrates clearly that the type II infection has been the rarest parainfluenza infection encountered in Copenhagen in recent years

Occurrence of Antibodies against the RS Virus Complement-Fixing Antigen

Fig 5 shows the distribution according to age of 404 paired sera examined and of sera containing antibodies against this antigen The percentage of sera containing antibodies increases from approximately 5 per cent in the age group 0 years to approximately 50 per cent in the age group 4 years This is also shown in Fig 6 In the age groups above 4 years the percentage of sera containing antibodies shows a tendency to decline As was the case with the percentages of sera showing antibodies against parainfluenza type II antigen, this decrease in the older age groups may also be fallacious where this antigen is concerned, because of the comparatively small number of sera examined in these age groups No definite difference in the frequencies of paired sera showing more than a two fold increase of titre could be found for the age groups examined, as is apparent from Fig 5

Results of Tests for Influenza A, B, C and Adeno Virus Complement Fixing Antibodies

At the Influenza department 473 paired sera have been examined for these antibodies as described above No increase of titre was found against influenza A and only one increase of titre, in a patient with a lower respiratory tract infection, against influenza B Six patients showed an antibody response to influenza C and 15 patients showed an antibody response to adeno virus C–F antigen The percentages of paired sera showing presence of antibodies against the last mentioned antigen in one or both sera (dilution 1 4 being the lowest serum dilution examined) are shown for the age groups examined (0 9 years) in Fig 6 It can be seen that there was an increase from approximately 35 per cent in the age group below 1 year to approximately 60 per cent for the age groups above 3 years

Results of Tests with Mumps and Ornithosis Complement-Fixing Antigens

272 paired sera were also tested for C–F antibodies against ornithosis but no presence of antibodies was found Because of the known antigenic relationship between parainfluenza viruses and mumps virus, 23 of the 31 paired sera which showed antibody responses to parainfluenza virus antigens were also tested with mumps antigen, but no concomitant increases of titre were found

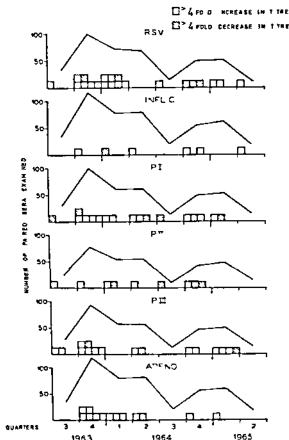


Fig 7

Distribution according to season of paired sera showing C–F antibody responses to RS, influenza C parainfluenza I II and II and adeno viruses Total numbers of sera examined for antibodies to the mentioned viruses in each of the quarters of the period September 1963 to May 1965 are indicated

Distribution of Positive Sera According to Season

The blood samples examined were collected over a 20 month period from September 1963 to May 1965 as mentioned above Paired sera showing more than a two fold increase of titre against one of the C–F antigens mentioned above have been divided according to the quarter in which the first blood sample was obtained This is illustrated in Fig 7, where each pair of sera which gave an increase of titre of four-fold or more against one of the antigens indicated is represented by a small equare The six graphs in Fig 7 each show the total number of sera examined in each quarter of the 20 month period against the various C–F antigens which are from above 1) RS, 2) Influenza C 3) Parainfluenza I 4) Parainfluenza II, 5) Parainfluenza III and 6) Adeno The sera which showed a four fold or greater decrease of titre

TAB

Distribution According to Main Diagnosis of Posit and Examined for Different Vi

Diagnosis	RSV		P I		Patients examin
	N exam *	Pos †	N exam	Pos	N exa
Pneumonitis	196	13 (6 6)§	193	7 (3 6)	16
Bronchopneumonitis	26	2 (7 7)	25	1 (4 0)	2
Bronchiolitis	1	0 –	1	0 –	
Acute Bronchitis	38	2 (5 3)	40	0 –	3
Acute Laryngitis	11	0 –	12	0 –	
Acute Rhinopharyngitis	10	0 –	10	0 –	
Catarrhalia	15	0 –	15	0 –	1
Acute Tonsillitis	11	1 (9 1)	9	0 –	
Acute Supp Otitis Media	12	0 –	10	0 –	
Febrile Convulsion	18	0	15	0 –	1
Others	66	0	68	3 (4 4)	5
Total	404	18 (4 5)	398	11 (2 8)	32

* N exam = Number examined

§ *Numbers in brackets indicate percentage of paired sera examined from patients wi*

against one of the mentioned antigens are represented by the hatched squares These also assist in the evaluation of which season the different respiratory infections might show the highest prevalence As can be seen from Fig 7, there is some accumulation of RS infections (top graph) and of adeno infections (bottom graph) in the winter months As it is evident from these graphs that a larger number of sera have been examined in the winter months, these accumulations might only be apparent No seasonal accumulation of parainfluenza or influenza C infections could be found This is evident from Fig 7, which also demonstrates higher frequencies of parainfluenza I and III infections than of parainfluenza II infections

Distribution of Sera Examined and of Increases of Titre Obtained According to Diagnosis

The examined sera were divided according to the different diagnoses shown in the column to the left in Table 1 These diagnoses represent the main diagnoses attached to the patients at the time of discharge from the hospials "Others" comprise various neurological, haematological and urinary tract disorders, but both in this group and in the group listed as "febrile convulsion" the major part of the patients had a respiratory infection as a secondary diagnosis In the group listed as acute laryngitis a few of the patients had the diagnosis acute laryngo tracheobronchitis Of the 473 patients examined for influenza C or adeno virus infections only 41 did not have a respiratory tract infection That is 91 per cent of the patients examined had either an upper or a

era from Children Hospitalized in Copenhagen 1963–65
nfections by Complement Fixation Test

or (infection)

I	P III		Infl C		Adeno	
Pos	N exam	Pos	N exam	Pos	N exam	Pos
3 (1 8)	178	7 (3 9)	238	3 (1.3)	238	11 (4 6)
1 (4 5)	25	1 (4 0)	26	2 (7 7)	26	0 –
0 –	1	0 –	1	0	1	0 –
0 –	36	1 (2 3)	41	0	41	0 –
0 –	12	2 (16 7)	17	0 –	17	1 (5 9)
0 –	10	0 –	11	0	11	1 (9 1)
0 –	13	0 –	16	0	16	1 (6 3)
1 (12 5)	9	0	11	0 –	11	1 (9 1)
0 –	7	0 –	12	0 –	12	0 –
0 –	13	0 –	19	0	19	0 –
1 (1 7)	64	3 (4 7)	81	1 (1 2)	81	0 –
6 (1 8)	368	14 (3 9)	473	6 (1 2)	473	15 (3.2)

† Pos = Number of positives (≥ four fold increase of titre)
the diagnosis in question

lower respiratory tract infection It is evident from Table 1 that the majority of patients examined had a lower respiratory tract infection

Of the 70 paired sera showing an increase of titre to one of the antigens listed in Table 1, only four showed a double increase to two of the antigens Three showed a double increase to parainfluenza type I and type III antigen and are listed as type I infections in Table 1 The fourth showed a double increase to parainfluenza type II and type III and is listed as a type II infection in Table 1

Table 1 demonstrates that no major difference in percentages of positive sera in the different categories of diagnoses listed could be found Comparison of these percentages is furthermore made difficult by the differences in the number of sera examined A number of sera in the group listed as "others showed increase of titre to the parainfluenza antigens or to the influenza C antigen With exception of the increase seen to the influenza C antigen all other increases of titre in this group appeared in patients who had a respiratory tract infection as a subdiagnosis

In Table 2 all sera from patients with a respiratory tract infection as main diagnosis have been divided according to the principal localization of the infection upper or lower The percentages of positive sera (indicated in the brackets obtained with the different antigens employed only showed a difference between upper and lower respiratory tract infection when the RS antigen was used The results suggest that the RS virus infection was mainly associated with lower respiratory tract infection in the children examined

TABLE 2

Distribution According to Localisation of Respiratory Disease of Paired Sera from Children Hospitalized in Copenhagen 1963–65 with Respiratory Infections The Number of Sera Examined and Number of Sera Giving a Positive Reaction in Complement Fixation with the listed Antigens Are Indicated

Examined for	Upper respiratory infections			Lower respiratory infections		
	N exam *	Pos †	%	N exam	Pos	%
RS	86	1	1.2	279	17	6.1
P I	85	1	1.2	277	10	3.6
P II	61	2	3.3	234	4	1.7
P III	79	3	3.8	256	11	4.3
Infl C	102	0		330	5	1.5
Adeno	102	4	3.9	330	11	3.3

* N exam = number examined
† Pos = Positives ≥ four fold increase of titre

TABLE 3

Distribution According to Age of Paired Sera from Children Hospitalized in Copenhagen 1963–65 with Pneumonitis of Bronchopneumonitis Examined by Complement Fixation Test with the Various Respiratory Virus Antigens Listed

Examined for	Number of positive sera (> 4 fold increase of titre) Age groups (years)					
	0	1	2–4	5–9	0–9	%
RSV	2/78*	4/54	6/71	5/51	17/254	6.7
Infl C	0/103	3/67	2/79	0/54	5/303	1.7
P I	1/79	2/49	5/80	1/49	9/257	3.5
P II	1/65	1/42	1/71	3/42	6/220	2.7
P III	2/68	5/47	2/78	2/46	11/239	4.6
Adeno	7/103	2/67	2/79	1/54	12/303	4.0

* The numbers to the right of the oblique line indicate number of sera examined with the virus antigen in question in the age group concerned

It can be seen from Table 1 that a major part of the sera examined were from patients with diagnoses of pneumonitis or bronchopneumonitis. Also some sera in the groups named "febrile convulsion" or "others" in this Table originated from patients with a subdiagnosis of of pneumonitis or bronchopneumonitis. In Table 3 all sera from patients with these two diagnoses are divided according to age of the patients. The number of sera examined and the number of positive sera found in each group with the six antigens in question are presented in this table. Some slight accumulation of positive sera in 1–4 year old patients examined with influenza C or parainfluenza I antigen and in patients below one year of age examined with adeno virus antigen can be seen. Somewhat different numbers of paired sera were examined with the different antigens. When this fact is kept in mind it is estimated that approximately a quarter of children with pneumonitis or

bronchopneumonitis were infected with either RS virus influenza virus type C, parainfluenza virus or adeno virus as determined by complement fixation tests These viruses were associated with approximately 6–7 per cent, 1 5–2 per cent, 11–12 per cent, and 3–4 per cent of these infections respectively

DISCUSSION

Evidence of parainfluenza infection demonstrated either by recovery of the viruses or serologically has established the endemic nature of these viruses (5 30) A survey showing the percentages of complement fixing antibodies to these viruses in different age groups of children should therefore give an estimate of the prevalence of parainfluenza infection in the different age groups, together with the relative importance of the different main types of these viruses As shown in this paper the percentages of children who possess antibodies to these viruses increase sharply during the first four to five years of live The same increase of percentages of children who possess antibodies have been demonstrated by other authors who either tested for the presence of neutralizing antibodies (10, 30) or for the presence of haemagglutination (HI) antibodies (23 35) The percentages of neutralizing antibodies reported in the different age groups (10, 30) are very similar to analogous percentages for complement fixing antibodies reported in this paper The finding that neutralizing antibodies to parainfluenza III appear at an earlier age and are more frequently encountered in all age groups examined than neutralizing antibodies to parainfluenza I and II (10, 30), is also confirmed when complement fixing antibodies are tested for, as shown in the present report

The distribution according to age of the percentages of sera showing presence of complement fixing antibodies to the RS virus given in this paper is almost identical to a similar distribution reported by *Vuvell & Deibel* (43) but the percentages are lower than those in a similar distribution of antibodies to this virus reported by *Hambling* (14) The distribution according to age given in this paper shows somewhat higher percentages of sera containing antibodies in the age groups concerned (0 9 years) than those which have been reported previously by the author (17) On the other hand in this previous report (17) the sera forming the basis for the age distribution of antibodies were collected from the whole of Denmark Furthermore it was pointed out (17) that the percentages of sera containing antibodies in the age groups concerned (0 9 years) might vary in successive years

An incidence of sera containing antibodies to adeno virus complement fixing antigen increasing from 3[illegible] per cent in the age group 0 year to approximately 60 per cent in the age group 3 years has been reported in this paper This incidence corresponds closely to similar incidences given by *Potter & Shedden* in England (32) and by *Jordan*

in America (22) and confirms the high prevalence of these infections in infancy and early childhood

The epidemic pattern of influenza A and B and RS virus infections as opposed to the more endemic nature of adeno and parainfluenza virus infections is now well established (5, 16, 34, 36), even though small circumscribed epidemics have often been encountered with the two last mentioned kinds of viruses The low incidence of parainfluenza infections during all seasons found by other authors (30) has been confirmed by the findings described in this paper All three types of parainfluenza infections were encountered during all seasons in Copenhagen without any accumulations Severe lower respiratory tract infections in children caused by adeno viruses or RS virus are accountered most often during the winter and spring (6, 16, 40) In our serological survey we have encountered RS virus infections and adeno virus infections most often during the months October to March As we have tested most sera in these months this finding might be fallacious, as mentioned above

Several serological studies have supported the great importance of the RS virus as aetiological agent for severe respiratory disease in infancy and early childhood (9, 13, 25, 28, 29) *Parrott et al* (29) found most increases of titre by complement fixation in infants 7–12 months of age in a large group of patients with severe respiratory disease, but could find no association between RS infections and any particular age group in patients with mild respiratory disease We found most RS infections in patients with lower respiratory tract disease, but could not find any association with a particular age group The proportion of cases of respiratory disease which were found to be associated with RS infection in the present survey was lower than found by others (25, 29) but this fact may be explained by a number of circumstances RS virus is a very important agent in the bronchiolitis syndrome (9, 13, 29), which was only diagnosed in one patient is our survey This could of course be a question of difference in the clinical terminology employed More important is the fact that there was only an interval of approximately 10 days between the blood samples in the majority of pairs of sera examined According to *Ross et al* (33) results of complement fixation tests with RS virus antigens were improved by postponing the collection of the convalescent serum until the fourth or fifth week after the start of illness On the other hand we did titrate our RS antigen against a pool of sera from small children as recommended by these authors (33) Even when sufficient consideration is given to technical problems the complement-fixation for RS virus is still probably somewhat inefficient in early infancy (4) and a large part of the patients in the present survey belonged to this age group This moderate sensitivity of the complement-fixation test for the diagnosis of RS virus infection in early infancy and in childhood (20 30 per cent and 80 90 per cent respectively) (4 29) must be taken into consideration when

the results obtained are evaluated. Therefore it is estimated that RS virus have been responsible for approximately 10–11 per cent of the pneumonitis and bronchopneumonitis infections investigated and not only 6.7 per cent as listed in Table 3.

The percentage of respiratory disease in children in Copenhagen found to be associated with parainfluenza infection as diagnosed by complement fixation, 9–10 per cent (Table 2), is similar to results obtained by this method in Germany (44), in Holland (3), and in America (15). About 70–80 per cent of infants and children infected with parainfluenza viruses have been reported to show homotypic and/or heterotypic antibody responses by complement fixation (30). Therefore it is estimated that approximately 14–15 per cent (and not 10–11 per cent as listed in Table 3) of the patients with pneumonitis or bronchopneumonitis have been infected with parainfluenza viruses. We were unable to confirm the close association between parainfluenza type I infection and the croup syndrome found by other laboratories (2, 26), but the number of patients with this syndrome in our survey was rather small. According to *Parrott* (31) parainfluenza type II plays a larger part in the aetiology of bronchopneumonitis, bronchiolitis and acute bronchitis in children than does parainfluenza type I. We were unable to confirm this, even though only three sera gave a double increase of titre with parainfluenza I and III antigens and were listed as parainfluenza I infections, as mentioned above.

The close antigenic relationship between the different parainfluenza viruses makes a specific aetiological diagnosis by complement fixation with these antigens uncertain (3, 30). In the present survey an increase of titre to P III antigen was seen in one (17 per cent) of the demonstrated increases of titre to P II antigen and in three (27 per cent) of the demonstrated increases of titre to P I antigen. Even so these antigens supplement one another in the establishment of the (unspecific) diagnosis of parainfluenza infection by serological means. Proper consideration must also be given to the antigenic relation of the parainfluenza viruses to the mumps virus in the evaluation of the results of a serological survey with the former viruses (8), even though this relationship is closer in sera from adults than from children (24). No double increases of titre to parainfluenza and mumps antigen were found in this survey, as mentioned above.

It has been estimated that adeno viruses are responsible for a few per cent of respiratory disease in civilian populations (22, 41). This is in accordance with the results presented in this survey. *Vargosko et al* (42) have reported that 19 [illegible] per cent of 22[illegible] infants and children from whom adeno viruses were recovered showed a complement fixing antibody response. These authors found that the highest percentage of antibody responses occurred in infants between the ages of [illegible] and 12 months and that antibody responses occurred almost exclusively in infants and children who were ill. In this survey most of the increases

of antibody were also seen in the age group below 12 months It was reported (42) that 39 per cent of the patients who were ill and from whom adenoviruses had been recovered from a throat swab specimen showed an antibody response In this paper 3–4 per cent of patients with upper or lower respiratory infections showed an antibody response to adeno virus complement-fixing antigen (Table 2) If the complement-fixation employed in this report have been of similar sensitivity as the test employed by the authors referred to above (42) then it can be estimated that a least 6–8 per cent of infants and children hospitalized in Copenhagen 1963–65 with respiratory infections have had an adeno virus infection Several reports from recent years (7, 11, 20, 21, 38) have established the adeno viruses as important aetiological agents in severe lower respiratory tract infections especially in cases of pneumonitis in infancy and childhood *Jansson & Wager* (19) examined 517 patients (all ages) with pneumonitis or bronchopneumonitis for complement-fixing antibodies to adeno type 2 antigen Of these 517 patients 5 per cent showed a significant antibody response and half of the higher antibody titres were seen in the age group 1–5 years In the present study there was no accumulation of adeno virus antibody responses in lower respiratory infections and no association between antibody response and any particular disease syndrome could be demonstrated

Influenza C virus is usually considered to be responsible only for cases of mild upper respiratory tract disease (27) Occasionally this virus may be associated with more severe respiratory disease in children *Wenner et al* (46) found influenza C to be an associated cause in four of 42 infants with either bronchiolitis or pneumonitis In the present survey antibody responses to influenza C were found in five of 264 cases of pneumonitis or bronchopneumonitis, all of which were in the age group 1–4 years

SUMMARY

Infants and children aged 0 9 years, admitted to hospitals in Copenhagen 1963–65 mainly for respiratory tract illnesses have been examined for complement fixing antibodies to influenza A, B, C, parainfluenza I, II, III, adeno and respiratory syncytial viruses The majority of the patients were also tested for complement-fixing antibodies to ornithosis

Parainfluenza III infections were found to be more prevalent than parainfluenza I and II infections RS and adeno virus infections were mainly diagnozed during the months October to March while no seasonal variations were observed for the parainfluenza infections A majority of the diagnozed RS infections were seen in patients with lower respiratory tract infections, while no preference of localization was found for adeno parainfluenza or influenza infections A majority of the sera examined came from patients with diagnoses of pneumonitis

or bronchopneumonitis RS influenza C, parainfluenza and adeno viruses were found by the complement-fixation tests employed to be associated with approximately 6–7 per cent, 1–2 per cent, 11–12 per cent and 3–4 per cent of these infections respectively From the sensitivities found by other authors for complement-fixation tests employed in the diagnosis of RS, parainfluenza and adenovirus infections it is estimated that these infections have been aetiological factors in 10–11 per cent, 14–15 per cent and in 6 8 per cent of the pneumonitis or bronchopneumonitis infections examined

No antibody responses were found to ornithosis or influenza A Only one antibody response were found to influenza B

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MYCOPLASMA PNEUMONIA INFECTION IN INFANTS AND CHILDREN IN COPENHAGEN 1963–65

Incidence of Complement-Fixing Antibodies in Age Groups 0–9 Years

By

ALLAN HORNSLETH

Received 29 vii 66

Several reports have established the importance of *Mycoplasma pneumoniae* (Eaton agent) as aetiological agent in acute respiratory tract disease in military and adult civilian population groups (2, 6, 10, 11, 12, 17, 24, 26), but few have been concerned with the possible importance of this agent in respiratory tract disease in childhood (2, 5, 17, 21) Complement fixation with dissimilar antigens was employed by *Thomas et al* (25) in the study of cases of primary atypical pneumonia (PAP) *Liu et al* (22) described the development of specific antibodies to the Eaton agent in PAP patients, they employed the fluorescent antibody technique The growth of the Eaton agent on artificial media was accomplished by *Chanock et al* (7) who also described the method for preparation of complement fixing antigen (8) Other methods for preparation of this antigen have been given by *Jansson* (15) and by *Kenny* (18)

Complement-fixing antibody responses to *Mycoplasma pneumoniae* (*M pn*) have been determined in infants and children admitted to hospitals in Copenhagen over a 20 month period The percentages of children showing presence of antibodies and the incidences of increases of titre for the age groups 0–9 years have been established

MATERIAL AND METHODS

The material consists of 367 paired sera collected from paediatric departments in hospitals in Copenhagen during the period September 1963 to May 1965 These sera form part of a number of sera which have been examined for complement fixing antibodies against different respiratory viruses as described elsewhere (14) Approximately 90 per cent of the sera originated from patients whose main diagnosis was a respiratory tract infection

The technique of *Bradstreet & Taylor* (3) was employed in all complement fixation tests All the specimens were heated for 30 minutes at 56° C and stored at —20° C until examined Control antigen and both positive and negative sera were included as controls in all test series

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The author is indebted to Dr K *Lind* the *Department of Toxoplasmosis and Viral Diseases Statens Seruminstitut* Copenhagen for carrying out tests for antibodies against *Mycoplasma pneumoniae* by the indirect fluorescent antibody technique

Antigen employed *M. pn.* complement fixing (C-F) antigen made by Robbin Laboratories Inc., Chapel Hill, N.C., U.S.A. The antigen was titrated against a positive serum with a fluorescent antibody titre of 1250 kindly supplied by Dr. A. *Lind*, Statens Seruminstitut.

The fluorescent antibody tests (FAT) performed at the department of Toxoplasmosis and Viral diseases, Statens Seruminstitut, have been described (21). Cold agglutinin titrations were also carried out in this department as described previously (20).

The methods employed in C-F tests with influenza, parainfluenza, adeno and respiratory syncytial viruses have been described elsewhere (14).

EXPERIMENTAL

Distribution According to Age of C-F Antibodies against Mycoplasma pneumoniae

Table 1 shows the distribution according to the patients' age of the 367 paired sera examined. It can be seen that antibody was present in the majority of sera from patients over the age of 11 months. Even though the sera examined constitute a very selected material, infection with *M. pn.* must have been very prevalent in children in Copenhagen during the last few years. The percentages of sera with a four fold increase of titre (the column to the right in Table 1) show a gradual increase with age, but the small numbers of sera examined in elder age groups do not allow any definite conclusion to be drawn in this respect.

TABLE 1

Distribution according to Patients' Age of 367 Paired Sera Examined for C-F Antibodies to Mycoplasma pneumoniae

Age group	No. tested	Antibody titre ≥ 8 in one or both sera		≥ 4 fold increase in titre	
		No.	per cent	No.	per cent
0–5 months	41	4	9.8		
6–11 "	80	33	41.5	3	3.8
1 year	67	47	0.1	1	1.5
2 years	46	35	76.1	1	2.2
3 "	34	22	64.7	2	5.9
4 "	29	14	63.6		
5 "	20	14	0.0	3	15.0
6 "	21	19	90.5	2	9.5
7 "	14	10	71.4	2	14.3
8 "	17	15	88.?	2	11.8
9 "	5	4	80.0	1	20.0

Distribution According to Diagnosis of C-F Antibodies to Mycoplasma pneumoniae

This distribution is shown in Table 2. The diagnoses listed to the left in this table represent the main diagnoses attached to the patients at the time of discharge from the hospitals. Others comprising various neurological, haematological and urinary tract disorders, 37 o... n 6

sera examined in the group listed as "others" and 14 of the 15 sera examined in the group listed as "febrile convulsion" came from patients with a respiratory tract infection as a secondary diagnosis That is, 92 1 per cent of the 367 patients examined had a respiratory tract infection From Table 2 can be seen that five of the 17 patients who showed a significant antibody response belonged to the group listed as "others" Of these five two had a pneumonitis and two had an upper respiratory tract infection as a secondary diagnosis, whilst the fifth had a main diagnosis of anaemia, but no respiratory tract infection That is 14 (82 per cent) of the 17 significant antibody responses came from patients with a lower respiratory tract infection, but it must be stressed that these patients contributed the majority of sera examined, as is evident from Table 2 Of 338 patients with respiratory tract infections 16 or 4 7 per cent showed a significant antibody response to the *M pn* C-F antigen employed Table 2 reveals furthermore that a large part of the sera which showed increasing, decreasing or high non-shifting antibody titres came from patients with clinical diagnoses of viral penumonitis or primary atypical pneumonitis

TABLE 2

Distribution According to Main Diagnosis of 367 Paired Sera from Children Hospitalized in Copenhagen 1963–65 Examined for Antibody Responses to Mycoplasma pneumoniae *by C F Tests*

Diagnosis	Number of sera examined	≧ 4 fold increase in titre	≧ 4 fold decrease in titre	Antibody titre* ≧ 512
Bronchopneumonitis	23	1 (4 3)†	0	1 (4 3)
Pneumonitis	112	3 (2 7)	0 –	2 (1 8)
Viral pneumonitis	27	1 (3 7)	2 (7 4)	4 (14 8)
Primary atypical pneumonitis	38	6 (15 8)	1 (2 6)	3 (7 9)
Acute bronchitis	36	1 (2 8)	0	0 –
Bronchiolitis	1	0 –	0	0 –
Acute laryngitis	9	0 –	0 –	0 –
Acute rhinopharyngitis	8	0	0 –	0 –
Catharralia	14	0 –	0	0 –
Acute tonsillitis	9	0 –	0	0 –
Febrile convulsion	15	0	0 –	0 –
Acute supp otitis media	10	0	0	0 –
Others	65	5 (7 8)	0	0 –
Total	367	17 (4 6)	3 (0 8)	10 (2 7)

* No increase or decrease in titre
† Numbers in brackets indicate percentage of sera with the diagnosis in question

Relationship between C F Antibody Response to Mycoplasma pneumoniae *and Corresponding Cold Agglutinins*

313 of the 367 paired sera examined for C-F antibodies were also examined for cold agglutinins (CA) According to the test employed for determination of CA (20) a titre ≧ 64 or a four-fold increase of

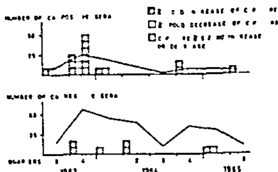


Fig. 1

titre was considered positive. Of the 313 sera examined 238 were found to be CA negative whilst the remainder (75 sera) were found to be CA positive. In Fig. 1 is shown the distribution according to season of these CA negative and CA positive sera. Each of the paired sera showing an increase or a decrease of titre together with sera which gave a titre ≧ 512 without decrease or decrease of titre in C-F tests are indicated by a little square. Most of the sera which were found by C-F tests to be associated with a *M. pn.* infection were CA positive. In the fourth quarter of 1963 11 out of 25 CA positive sera (44.0 per cent) were associated with a *M. pn.* infection but only 2 out of 56 CA negative sera (3.6 per cent) were associated with this infection as found by C-F tests. The variation according to season of the number of sera examined makes it difficult to say whether there was any seasonal variation in the number of sera which showed an increase or decrease of titre.

With the object of obtaining an estimation of how the C-F titres varied in comparison with the corresponding CA titres during the first few weeks following the start of illness, an analysis of 22 paired sera with high antibody titres, increase or decrease of antibody tiltres to *M. pn.* and with known CA titres was carried out. According to the time of blood sampling the 44 *M. pn.* C-F titres obtained together with the corresponding CA titres were divided up into groups each of which represented a period of one week. In this way five to 13 titres were included in each of four groups corresponding to the first four weeks following start of illness. The mean titres were calculated for each group and in Fig. 2 these weekly mean C-F and corresponding CA titres are shown in logarithmic form. Both the C-F titres and the CA titres seem to reach a maximum in the third week following start of illness but the CA titres seem to decline faster in the following weeks.

Relationship between Corresponding C-F and Fluorescent Antibody Responses

Table 3 shows the titres obtained in 14 paired sera examined for these two kinds of antibodies. With the fluorescent antibody test (FAT) em

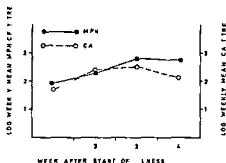


Fig 2

ployed (21) a four-fold increase of titre or a titre of ≧ 160 was considered positive It can be seen in Table 3 that there was a close correlation between the two kinds of antibodies The six pairs of sera which showed either high antibody titres or gave a four-fold or greater increase of titre by C–F test all gave a positive FAT The pairs of sera which showed either low or negative antibody titres by C–F test all gave a negative FAT

TABLE 3

Antibody Responses in 14 Paired Sera Examined for Antibodies to Mycoplasma pneumoniae by Fluorescent Antibody Test (FAT) and by Complement Fixation Test (C F)

Serum	FAT	C F
14657 I	40 (80)	16
– II	160 (320)	256
14840 I	≦40	64
II	320	>1024
14879 I	≦40	16
II	320 (640)	512
15518 I	80	32
– II	80 (160)	128
16634 I	40 (80)	8
II	160	64
16698 I	320	1024
– II	640	512
14816 I	≦40	32
II	≦40	32
15819 I	<40	32
– II	<40	16
16020 I	<40	32
II	<40	64
16653 I	<40	<8
– II	<40	8
16867 I	<40	<8
– II	<40	8
17035 I	<40	<8
II	<40	8
17033 I	<40	<8
II	<40	<8
16802 I	<40	<8
II	<40	<8

Double Infections with Mycoplasma pneumoniae *and Respiratory Viruses*

All 367 paired sera examined for C-F antibodies against *M pn* have been examined for C-F antibodies against influenza, adeno and respiratory syncytial viruses (14) and the majority of these sera have also been examined for C F antibodies against parainfluenza viruses (14)

Of the 30 paired sera which responded to *M pn* C-F antigen with an increase or decrease of titre or revealed a titre ≧ 512 as listed in Table 2, 24 have been examined for C F antibodies against all the different respiratory viruses mentioned above Nine sera with *M pn* C-F titres ≧ 512 and two sera which showed decreases of titre against *M pn* did not show any double infection against the viruses mentioned Of 13 sera which showed an increase of titre against *M pn* one showed an increase of titre to respiratory syncytial virus, one showed an increase of titre against influenza type B and four showed increases of titre against parainfluenza viruses, (one against type I, one against type III and two against both type II and type III) That is, 6 og 13 sera (46 2 per cent) with increases of antibody against *M pn* also showed double infections with respiratory viruses

The patient with a double infection of *M pn* and influenza type B was a three year old girl with a diagnosis of pyuria, but with a subdiagnosis of recurrent rhinopharyngitis Double infection with *M pn* and respiratory syncytial virus was seen in a seven year old boy with bronchopneumonitis Four girls with ages ranging from 9 months to 8 years had double infections with *M pn* and parainfluenza viruses 1) the patients aged nine months had bilateral pneumonitis 2) a patient aged six years had been admitted with convulsions and a rash and was under observation for epilepsy, 3) one patient aged eight years had a diagnosis of pyuria and a subdiagnosis of catharalia and 4) another patient aged eight years had the diagnosis of infectious mononucleosis and a subdiagnosis of primary atypical pneumonia

DISCUSSION

Several reports support the value and specificity of C F tests in the study of PPLO infections *Card* (4) employed an antigen made from a PPLO strain which was isolated from a genital infection when she examined different categories of patients and healthy population groups for C F antibodies She found the highest incidence of antibodies in patients who were attending venereal distase clinics *Lemcke* (19) studied the immunological responses in rats and mice which were inoculated with several different strains of PPLO She found a high degree of correlation between the [illegible] of serum C F antibody and the presence of PPLO in the tis[illegible] these animals She furthermore observed that the quantity [illegible] body increased with increasing dissemination of the infection in [illegible] animal (19) *Chanock et al* (8)

compared the C–F test with the FAT in the diagnosis of Eaton agent infection in marine recruits and found the C-F test to be approximaely 80 per cent as sensitive as the FAT Both *Goodburn et al* (11) and *Biberfeld et al* (2) found good correlation between the C–F test and the FAT in the diagnosis of *M pn* infections *Grayston et al* (12) examined 463 patients (all ages) with respiratory tract infections for infection with *M pn* When the C–F tests was employed in 40 of 57 patients from whom this organism was isolated an increase of titre was demonstrated in all patients who had a titre < 16 in the first blood sample The specificity of the C–F test employed in the present report is supported by the good correlation which was found between C–F titres and fluores cent antibody titres in the sera examined (Table 3) Furthermore the CA positive group of patients included most of the infections which could be associated with *M pn* by C–F test (Fig 1) This finding is in accordance with observations made by *Jansson et al* (17) and by *Grayston et al* (12)

The percentages of sera in different age groups showing presence of C–F antibodies to *M pn* given in this paper are higher than analogous percentages given by *Grayston et al* (12) Even though a smaller number of sera were examined by these authors in the age groups concerned, 0–9 years, the wide fluctuation in the prevalence of *M pn* (13) is a more probable explanation of this discrepancy

M pn infections have been diagnosed most often in older children and in adults when either isolation of the infectious agent or demonstration of increases of antibody titres have been employed in patients with respiratory tract illness (2, 12, 17, 21) *Jansson et al* (17) found the highest incidence of positive antibody responses by C–F tests in the age groups 6–30 years *Grayston et al* (12) found the highest isolation rate of *M pn* in patients aged 10–19 years, but found the C–F test to be a more sensitive diagnostic procedure than isolation of the agent *Grayston et al* (12) isolated 4 strains of *M pn* from 60 patients aged 0–9 years *Biberfeld et al* (2) diagnosed by C–F test and FAT 5 cases of *M pn* infection in a group of 13 patients aged 6–10 years, but no cases in a group of 14 patients aged 0–5 years *Lind* (21) isolated 10 strains of *M pn* from 19 patients aged 5 14 years old but none from 27 patients aged 0–4 years In the present survey 2 4 per cent of patients aged 0–4 years and 13 0 per cent of patients aged 5–9 years showed antibody responses to *M pn*, but the difference in number of sera examined in these two age groups, 290 and 77 respectively, makes an assesment of these percentages difficult *Chanock et al* (5) found increase of antibody by FAT in 10 per cent of 152 infants and children with lower respiratory tract illnesses

Chanock et al (6) found that 40 per cent of a group of recruits with respiratory illness who were found to be infected with *M pn* by FAT were CA positive Of 37 cases of respiratory illness (mainly pneumonitis) found to give positive antibody responses in C–F test and FAT by

Biberfeld et al (2) 17 were CA positive When 246 patients with pneumonia, all ages, were examined for C–F antibodies against *M pn* (*Jansson & Wager* 1964) approximately two thirds of the patients who gave a positive antibody response were found to be CA positive *Grayston et al* (12) reported that approximately 75 per cent of a group of patients (all ages) with respiratory tract illnesses that gave a positive antibody response by C F were CA positive whilst only 2 per cent of the C F negative were CA positive *Grayston et al* (12) also observed that a positive CA response was a more frequent finding among children who showed increase of antibody by C F test than among adults who gave a positive antibody response by C F As described in this paper the majority of patients who gave a positive antibody response by C F test were CA positive

Finland et al (9) described that maximum titres of cold agglutinins were seen between the 11th and 21th day following the start of illness (primary atypical pneumonia) and that titres decreased in the third or fourth week Similar observations concerning the cold agglutinin titres were made by *Jansson & Wager* (16) *Grayston et al* (12) found that maximum C F titres were reached in the beginning of the fourth week following start of illness and that the titres only decreased moderately in the following few months This temporal distribution of the C–F titres obtained seems to follow the pattern for fluorescent antibody titres as described by *Liu et al* (22) According to the results presented in this paper maximum C–F titres seem to be reached mainly in the third week following start of illness

Liu I (21) found evidence of double infection with *M pn* and adeno viruses in two out of 65 patients with pneumonia *Baernstein et al* (1) found increases of C-F antibody against the respiratory syncytial virus in two out of six children from whom *M pn* was isolated Some double infections with *M pn* and adeno viruses or influenza virus type A have been observed by *Marmion & Hers* (23) In the present paper concomitant infections with *M pn* and respiratory viruses were frequently observed Six out of 13 patients who showed increases of titre against *M pn* were also infected with a respiratory virus These double infections were most frequently seen with parainfluenza viruses but did not occur in any particular age group

SUMMARY

367 paired sera from infants and children aged 0 9 years admitted to hospitals in Copenhagen during the period September 1963 to May 1965 have been examined for C F [illegible] against *Mycoplasma pneumoniae* The majority of sera [illegible] ts over the age of 12 months showed presence of antibodie[illegible] nt of patients with respiratory tract infections showed signi[illegible] tibody responses The percentage of patients who showed anti[illegible] responses was higher in the age

group 5–9 years than in the age group 0–4 years The major part of patients who could be associated with a *M pn infection by C–F tests* were found among the group who showed presence of cold agglutinins (CA) Maximum C–F and CA titres were generally reached in the third week following the start of illness Of 13 paired sera which showed an increase of C–F titre six showed double infections with respiratory viruses

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activity was tested by filling the enzyme into 10 mm diameter holes cut in the agar and reading after 24 hours incubation at 37° C.

Spectrophotometric determinations Infrared absorption spectra were recorded using a Perkin Elmer spectrophotometer model 237 Samples for infrared analysis were prepared either as dry film on Irtran 2 cells or pressed into tablets with KBr in vacuo under a pressure of 10 tons per sq inch as recommended by Perkin Elmer Similar absorption bands were obtained by either method although the samples as KBr tablets gave a better resolution Extinction measurements on the fractions [illegible]

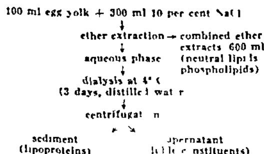
EXPERIMENTAL

It was demonstrated earlier (5) that the egg yolk reaction as studied by the agar plate method was composed of three phenomena, namely a clearing precipitation, and the formation of fatty material on the agar surface Repeated filling of the enzyme in the holes of egg yolk agar plates and incubation at 37° C overnight revealed a number of rings of clearing and precipitation

Egg yolk is a complex mixture of lipids, lipoproteins and other constituents, and the egg yolk reaction can be caused by more than one enzyme attacking different substrates in egg yolk Egg yolk was therefore fractionated in order to identify the substrate(s) which were involved

Identification of the Substrate(s) in Egg Yolk

The method described by *Chargaff* (1) for the preparation of lipoproteins was used for the fractionation of egg yolk Egg yolk was mixed with ten per cent sodium chloride, as outlined in the following scheme, and the free lipids were extracted with diethylether three times and after removal of the ether, the aqueous phase was dialysed to precipitate the lipoproteins which could be collected by centrifugation



The precipitated lipoproteins were redissolved in 10 per cent sodium chloride solution and precipitated again by dialysis and then taken up in 50 ml of sodium chloride solution Addition of a few drops of ether dissolved the turbidity to give an opalescent solution

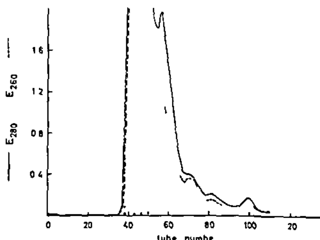


Fig 1
Separation of egg y lk lipopr teins n Sej h a lex (.00
(Dotte l region ind cates tl e locati n of the ti rl i l s l stance)

The different fractions derived from egg yolk were tested for substrate activity When mixed with the agar and poured into Petri plates the ether extract and the lipoproteins gave a turbidity whereas the agar plates cont uning the soluble constituents were clear No reaction w is obtained with the enzyme on j lates containing the ether extract and the water soluble fraction The lipoprotein fraction gave reactions similar to that obtained with unfractionated egg yoll yielding the clearing and precipitation phenomena as well as appearance of fatty material on the surface Thus the substrate was located in the lipo protein fraction which was further fractionated by gelfiltration using Sephadex G 200 gel (Pharmacia Uppsala)

Chromatography of lipoproteins on Sephadex G 200 5 ml of the lipoprotein solution w is chromatographed on Sephadex G 200 with a bed size of 1 5 cm diameter and 120 cm length using 0 1M Tris HCl buffer of pH 8 0 and containing 1M sodium chloride The effluent w is collected in 3 ml lots in an automatic fraction collector Extinction measurements at 260 and 280 mμ on the fractions gave the distribution pattern indicated in Fig 1 The first fractions were turbid and the turbid material tended to rise to the surface on standing Centrifugation at 3,000 g collected the turbid material as a sheet on the surfac leaving a clear yellow bottom phase

The different fractions were tested for substrate activity The contents of tubes up to number 65 when mixed with the agar gave a turbid suspension The clear yellow phase obtained on centrifugation also gave a turbidity on mixing with agar It was found that the turbid substance which was collected on the surface was the active fraction whereas the clear yellow solution was devoid of activity The turbid substance dissolved on the addition of a few drops of ether These properties of the

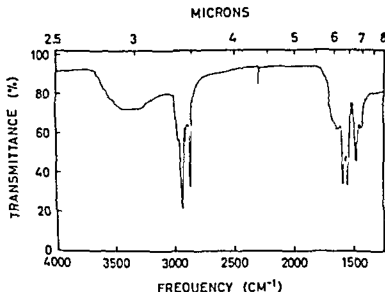


Fig 2
Infrared absorption spectrum of the fatty material from egg yolk agar plates (KBr pellet)

turbid substance corresponded to the description given in literature (2) for lipovitellenin and the clear yellow solution apparently contained lipovitellin The substrate active in the egg yolk reaction was thus identified as lipovitellenin

Identification of the Products of the Egg Yolk Reaction

The egg yolk reaction was composed of clearing and precipitating reactions with the liberation of fat appearing over the precipitation region on the agar surface The precipitate and the fat which apparently represented the end products of the egg yolk reaction could be easily removed with the aid of a nickel spatula and suspended in acetone No such material could be removed from the agar surface which had not reacted with the enzyme

Infrared absorption spectrum and melting point The fatty material and the precipitate collected from several egg yolk agar plates after reaction with the enzyme were pooled and examined for absorption in the infrared region The infrared spectrum of the material (Fig 2) showed the presence of a weak bonded $-OH$ (2 9–3 25 μ), R $C-H_3$ (3 42 μ), R-$C-H_2$ (3 5 μ) and C–H deformations in the region of 6–7 μ possibly with a C=C stretching weak absorption at 6 6 05 μ) The absorption was not characteristic of any specific substance but seemed rather to be a mixture Microscopic examination showed that it consisted of an oily substance and an amorphous material, the latter melt

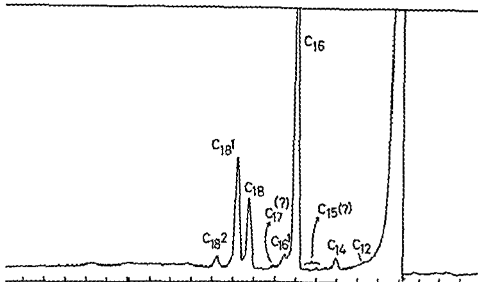


Fig 3

Gas chromatography of the fatty acids isolated from egg yolk plates after reaction with enzyme

ing at 196–201° C After treatment with hydrochloric acid, extraction with chloroform and evaporation of the chloroform phase, about 7 mg of the substance was obtained This contained molten and crystalline solids the melting points of which were at 25, 31–33 and 46–51° C, thus indicating the presence of not less than three major components

Analysis of fatty acids by gas chromatography The fatty acids were analysed by gas-liquid chromatography after boron trifluoride methylation Experiments done with an Aerograph and a Perkin Elmer 800 gas chromatographic instruments gave similar fatty acid distributions As shown in Fig 3, the major components in decreasing order were palmitic acid (C_{16}), oleic acid ($C_{18}1$) and stearic acid (C_{18}) The quantitative distribution of these and other fatty acids were as follows

myristic acid	(C_{14})	1 % ± 0 5 %
palmitic acid	(C_{16})	63 % ± 2 0 %
palmitoleic acid	($C_{16}1$)	1 % ± 0 5 %
stearic acid	(C_{18})	12 % ± 1 0 %
oleic acid	($C_{18}1$)	22 % ± 1 5 %
linoleic acid	($C_{18}2$)	1 % ± 0 5 %
other acids		trace amounts

Thus, the end products of the egg yolk reaction were fatty acids which occurred as insoluble calcium or magnesium salts

DISCUSSION

The experiments described in this study relate to the identification of the substrate in egg yolk and the products of the egg yolk reaction

Fractionation of egg yolk and examination of the of the fractions for substrate activity showed that the substrate occurred in the lipoprotein fraction. At least two lipoproteins, lipovitellin and lipovitellenin, are known to occur in egg yolk (2). Lipovitellin was shown to contain more phospholipids and less triglycerides, whereas lipovitellenin is not as readily soluble in sodium chloride solution as lipovitellin but dissolves on addition of ether. Further, lipovitellenin has a lower density than lipovitellin. The results obtained in the present study point to lipovitellenin as being the substrate active in the egg yolk reaction. The formation of fatty acids from lipovitellenin by the staphylococcal enzyme indicated that the latter was a lipoprotein lipase. The lack of reaction with lipovitellin was probably due to its lower content of triglycerides. It has been found that the staphylococcal enzyme is not active against phospholipids such as lecithin, cephalin and sphingomyelin (3, 8, 10). It thus seems that the fatty acids were liberated from the triglyceride part of lipovitellenin. *Shaw & Wilson* (8) also showed that it was lipovitellenin in egg yolk which acted as the substrate in the egg yolk reaction.

The fatty acid distribution indicated that palmitic acid occurred in high amounts followed in decreasing order by oleic and stearic acids. The proportion of the fatty acids reported in the present study did not correspond with the fatty acid distribution described by *Martin et al* (6). However, the latter writers hydrolysed the total lipids extracted from lipoproteins by chemical means and thus also included the phospholipids. The results obtained in the present study may be due to the relative rates at which the different fatty acid ester bonds were attacked by the enzyme. The greater occurrence of palmitic acid would point to a higher affinity of the enzyme to this ester than to those of oleic or stearic acid esters. A similar observation was also made by *Vadehra & Harmon* (11) during studies on the action of staphylococcal lipase on milk fat.

SUMMARY

The nature of the egg yolk reaction given by staphylococci was studied by fractionating egg yolk to identify the substrate and by analysing the products of the reaction. The substrate in egg yolk was identified as lipovitellenin from its low density and ready solubility in the presence of ether. The fatty acids which occurred as insoluble calcium or magnesium salts caused the precipitation reaction. Analysis of the fatty acids gave the following distribution: palmitic acid (63 per cent), stearic acid (12 per cent) and oleic acid ([illegible] per cent).

The egg yolk reaction was caused by a lipoprotein lipase acting on lipovitellenin.

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Acta path et microbiol scandinav 69 321 331, 1967

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NON-SPECIFIC IMMUNOFLUORESCENCE STAINING AND ITS ELIMINATION

Studies on Lymphoid Cells from Rabbits Immunized with Human Gamma Globulin

By

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Received 15 viii 66

Immunofluorescence methods have been used to demonstrate antigens in tissue or cells and also to demonstrate antibodies present in lymphoid cells (reviewed by *Nairn* 1964) Non-specific staining reactions often complicate immunofluorescence studies Non-specific interactions between fluorescent material and the cells or tissues studied have attracted most interest Many investigators have reported success in avoiding the problem by purification of the fluorescent antisera in various ways—for instance, *Riggs et al* (1960), *Curtain* (1961), *Goldstein et al* (1961), *Frommhagen & Spendlove* (1962), *McDevitt et al* (1963), *Engelgardt* (1964), *Wolf et al* (1965)—or by special treatments of the cell or tissue preparations—for instance, *Mayersbach & Schubert* (1960) With the indirect fluorescence method staining due to non specific reactions by the unconjugated middle layer has attracted less interest, although discussed by *Myers et al* (1965) At a study with immunofluorescence methods on antibody content of lymphoid cells the latter type of non specific fluorescence became a serious problem and initiated a detailed investigation on such phenomena and on possibilities of eliminating them by various treatments

MATERIAL AND METHODS

Antigen A commercial human gamma globulin preparation (AB Kabi Stockholm) was used It is supplied as a 12 per cent solution in a 0 3M glycine bicarbonate buffer at pH 6 8 stated to contain 93 per cent γG and 0 5 1 per cent albumin An immuno electrophoretically purer γG preparation (Kabi 1348) was used for immunofluorescence and immunodiffusion in agar This preparation contained except for γG mere traces of albumin

This investigation was financed by grant from the *Swedish Multiple Sclerosis Foundation* from the *Swedish Medical Research Council* (project 12X 579 01) and from the *Swedish Cancer Society* (project 64 164) My thanks to Dr *John Sjöquist* Department of Clinical Bacteriology Umeå who prepared the rabbit gamma globulin

Immunization schedule Adult rabbits (2 3 kg) of mixed stock were used Four groups of rabbits were studied

1) Untreated called untreated controls—four rabbits

2) Injected with Freund s complete adjuvant (Difco), called adjuvant treated controls—nine rabbits One of these was given 0 5 ml intravenously and 0 5 ml intracutaneously in the paws The other eight were injected intracutaneously with 0 1 ml in equal volumes of saline The injections were repeated once after 9 10 days

3) Injected intracutaneously in the paws with 6 mg of human gamma globulin in saline emulsified with an equal volume of Freund s complete adjuvant—15 rabbits They were given 0 2 ml repeated after 9 10 days Some animals had repeated in jections (up to five) at varying intervals No material differences in results were seen between the former and the latter rabbits and most studies were performed on the former

4) Injected intravenously (10 or 20 mg per dose) and intraperitoneally (30 or 60 mg per dose) for a month according to the schedule suggested by *Boyd* (1956)—nine rabbits

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injected intravenously and intraperitoneally no antibodies could be demonstrated with this method some days after the last antigen dose Sera from all nine intra venously and intraperitoneally injected rabbits were therefore studied with a Coomb s test Human O Rh+ red blood corpuscules were coated with human anti D serum and tested for agglutination with the rabbit sera before and after the injection schedule The four rabbit sera that showed precipitates in the immuno diffusions had titres of 1 1024 or 1 2048 and the five rabbit sera without precipi tates were negative These five rabbits are called sero negative

Immunodiffusion in agar gel Rabbit sera before and after injection schedules were analysed with immunodiffusion in agar according to Ouchterlony s micro method with standard equipment from LKB (Stockholm) Undiluted serum was put in the central well and human gamma globulin in the peripheral wells in dilution steps of 1 2 in a wide range of dilutions

Preparation of lymphoid cells Cell suspensions were prepared from regional lymph glands and/or the spleen by teasing and the suspension filtered through double gauze and washed three times in saline A drop of the suspension was placed on a microscope slide This was put on a sand bath covered with a horizontal glass plate thermostatically controlled at 56° C and the drop of the suspension was dried and fixed in this manner for 20 minutes A cell density was chosen that permitted a relatively dense cell mat each cell however separated from its neighbours The slides were stored below —20° C

From some rabbits lymph glands have been removed more than once Therefore 11 cell preparations were studied from the nine adjuvant treated rabbits and 16 cell preparations from the 15 rabbits immunized intracutaneously with human gamma globulin

Reagents used at immunofluorescence Antiserum globulins conjugated with fluorescein isothiocyanate (FITC) were obtained from Microbiological Associates (Bethesda Maryland) goat antiserum globulin vs human serum globulin (control 49465) goat antiserum globulin vs guinea pig serum globulin (control 44315) Rhodamine conjugated bovine albumin (Microbiol Assoc) was used as counterstain in all conjugated antisera in recommended dosage Unconjugated goat antiserum vs human serum globulin was obtained from Microbiol Assoc (control 52213)

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Preincubation and blocking methods In some experiments human gamma glob ulin and conjugated antiserum globulin were preincubated with a pool of normal rabbit sera concentrated to a third of its original volume with a collodion bag (*Mies* 1953) This concentration was found suitable in preliminary experiments

One part of 12 per cent human gamma globulin and 11 parts of the pool were incubated for 90 minutes at 37° C. The conjugated commercial antiserum globulin was mixed with an equal volume of the pool and treated in the same way. Precipitates were not formed at any of these incubations.

In other experiments preincubation was performed with equivalent amounts of rabbit gamma globulin prepared by free electrophoresis in a 0.05M tris HCl buffer at pH 8.0. Paper electrophoresis showed it to be made up of 98.3 per cent gamma globulin and 1.7 per cent beta globulin.

In further experiments conjugated goat anti-human globulin was blocked with the antigen by incubation for two hours at 37° C. Two parts of the conjugate and one part of 12 per cent human gamma globulin were used. After incubation the mixture was centrifuged, one part of 12 per cent human gamma globulin was again added and incubation was continued for 12 hours at +4° C. After centrifugation preincubation as above with concentrate of pooled rabbit sera was performed. Parallel controls with 0.9 per cent saline instead of human gamma globulin were made.

Immunofluorescence method. Various modifications were used as further described under Results. The first step (incubation with antigen or control) was made with a volume of four drops at 37° C for 30 minutes. When intermediate step was used the slides were incubated with 5–9 drops of unconjugated goat serum at 37° C for 45 minutes; this procedure was repeated once or twice. The final step (conjugated antiserum globulin) was made at room temperature for 15 minutes with a volume corresponding to two drops of undiluted conjugate. As rinsing solution Sorensen's 1/15M phosphate buffer at pH 7.2 or saline buffered at pH 7.1 with 0.01M phosphate were used. The two solutions were found equivalent.

Microscopy was performed with a Zeiss fluorescence microscope equipped with an Osram high pressure Mercury arc lamp HBO 200, BG 3 and BG 12 exciter filters and barrier filters 44 and 50 ("cut off" at 500 mμ). A 40 × objective was used. The microscope could easily be switched to phase contrast. Photographs could be taken on 35 mm High Speed Ectachrome film EHB 135-20.

The slides were read blindly by two different observers. Usually a positive control was included in the series of slides studied.

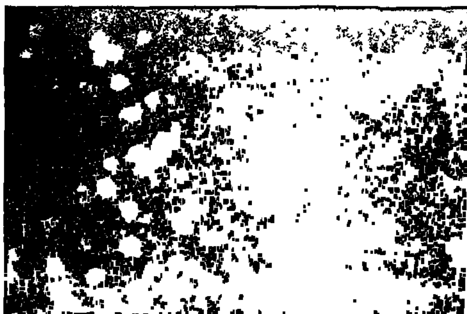
The fluorescence intensity was scored from (+) to ++. Positive slides are of two types. In one practically every cell is fluorescent with scores from (+) to +(+) or ++ (cf. Fig. 2). In the other type single fluorescent cells with a reaction of at least +(+) are observed in a negative (reddish brown) background (cf. Fig. 1). For further discussion on the scoring of positive slides see p. 32[illegible].

RESULTS

1. *Direct Fluorescence Method*

The simplest way to demonstrate the presence of cells with antibodies against human gamma globulin would be to use the direct immunofluorescence method. One per cent conjugated human gamma globulin in saline was added to slides from five rabbits: two untreated controls, one adjuvant treated control and two "sero-positive" rabbits. In all these slides a generalized fluorescence of equal strength was obtained (see Fig. 2).

Thus conjugated human gamma globulin becomes attached to cells of all [illegible] red to the cells from [illegible] between the human [illegible] ment of the human [illegible] possibly extinguish [illegible] the reaction. Human gamma globulin was preincubated with concentrated rabbit serum (cf. "Methods") and added to the five cel[illegible]



Figs 1 and 2

Fig 1 Colour photograph of lymphoid cells from a rabbit immunized intracutaneously with human gamma globulin Indirect fluorescence method first step human gamma globulin preincubated with normal rabbit serum *final step fluorescein-conjugated goat anti human globulin preincubated* with normal rabbit serum Four fluorescent cells scored (+) to ++ in a negative background

Fig 2 Colour photograph of lymphoid cells from a rabbit injected intracutaneously with Freund's complete adjuvant Direct fluorescence method treatment of slides with fluorescein-conjugated human gamma globulin Note generalized fluorescence

parations mentioned above No fluorescence could now be seen either in cells from controls or in cells from "sero-positive" rabbits. Thus either no specific attachment of human gamma globulin to cells from immunized animals occurred or the direct method used is too insensitive for its demonstration An indirect method was therefore tried

2 Indirect Fluorescence Method

Slides from three untreated and five adjuvant treated controls were exposed to one per cent human gamma globulin in saline in the first step and conjugated goat anti human globulin diluted in an equal volume of saline in the second A generalized fluorescence was obtained in all slides similar to that shown in Fig 2 This could not be due to a non specific attachment of FITC or FITC conjugated material to the cells as slides from these cell preparations were negative when exposed to saline in the first step without changing the second step The result indicates attachment of the unconjugated human gamma globulin to the cells in the first step

However FITC or FITC-conjugated material added in the second step may sometimes react with the cells This interaction varies in strength for different conjugates as well as for different cell preparations which the following experiments show Slides from two untreated and five adjuvant treated controls also studied with conjugated goat anti human globulin as described above were treated with human gamma globulin or saline in the first step and conjugated goat anti guinea pig globulin diluted in an equal volume of saline in the second Five cell preparations were negative but two showed single positive cells in a negative background both when the first step consisted of human gamma globulin and when it consisted of saline Among slides from three "sero-negative" rabbits studied with saline in the first step and conjugated goat anti human globulin diluted in an equal volume of saline in the second one showed a similar type of fluorescence resembling that shown in Fig 1

As it was demonstrated with the direct method that pretreatment of conjugated human gamma globulin with normal rabbit serum concentrate diminished or extinguished its absorption to the cells the same schedule was tried in the indirect method In the first step slides were treated with human gamma globulin that had been preincubated with concentrated normal rabbit serum In the final step conjugated goat anti human globulin in an equal volume of saline was used Slides from one untreated and two adjuvant treated rabbits all showed a generalized fluorescence

Although this generalized fluorescence was a little less pronounced than before this simple pretreatment obviously will not prevent non specific fluorescence Pretreatment with concentrated normal rabbit serum was therefore tried both in the first and the second step 20 ll

preparations from untreated controls, adjuvant-treated controls or "sero-negative" rabbits were studied in this way—all were now negative The same result was obtained with these cell preparations when the first step consisted of concentrated normal rabbit serum and the second step of conjugated goat anti-human globulin, pretreated with normal rabbit serum concentrate Thus, control cell preparations were now negative

Subsequently, 20 cell preparations from "sero-positive" rabbits were studied in the same way After pretreatment with normal rabbit serum concentrate in both steps, scattered strongly fluorescent cells in a negative background (see Fig 1) were seen in 18 preparations One of the two negative preparations were obtained from a regional lymph node, removed less than one day after the booster dose, given on day 10 The other was obtained from a spleen removed five days after the last injection to a rabbit, immunized intravenously

19 cell preparations from "sero-positive" rabbits were studied with *concentrated normal rabbit serum* in the first step and conjugated goat anti-human globulin, pretreated with concentrated normal rabbit serum, in the second step 18 of these were negative, but in one, scattered fluorescent cells were observed on a negative background This preparation was obtained from regional lymph gland and spleen, removed four days after the booster dose, given on day 10 Further analysis of this cell preparation (see below, p 327) showed that this reaction is reasonably immunologic This can be explained by a supposed presence of antigen in the cells It is surprising that such findings were not obtained more frequently

Thus, in the main, cell preparations from controls are now negative and from "sero-positive" rabbits positive No generalized fluorescence is observed Positive slides show many strongly fluorescent cells on a negative background It should be stressed, that a positive reaction is scored when the fluorescent cells are fairly numerous and show a strength of at least + (+) Such single cell in a whole slide is judged to be without significance, like cells with a fluorescence of (+) up to + strength

3 *Specificity of Reactions*

The specificity of the positive reactions in cell preparations from "sero-positive" rabbits were now further studied using three different systems

In the first series of slides, a conjugated goat anti guinea-pig globulin was used in the final step instead of the anti-human globulin Preincubation with normal rabbit serum concentrate was performed with both the human gamma globulin of the first step and with the conjugate of the final step Negative results were obtained with 20 cell preparations from "sero positive" rabbits

In the second series of slides the conjugated goat anti human globulin was pretreated with an excess of human gamma globulin in order to block specific antibodies. This blocked conjugate was then used in the final step after preincubation with concentrated normal rabbit serum, the first step being treatment with human gamma globulin also preincubated with rabbit serum concentrate. Three "sero-positive" rabbits showed negative slides. When conjugate was "blocked" with saline instead of human gamma globulin, however, positive slides were obtained in identical preparations.

In the third series the slides were first treated in the usual way with human gamma globulin preincubated with rabbit serum concentrate. An intermediate step was then made with unconjugated goat anti human globulin, followed by a final step of treatment with conjugated goat anti human globulin preincubated with normal rabbit serum concentrate. Three "sero-positive" rabbits, the same as used in the second series mentioned above, showed negative slides in this system.

The third system, however, might have been complicated by a non specific blocking effect of the goat serum in the intermediate step. Therefore a control series was run with the same cell preparations using normal goat serum in the intermediate step, the rest of the experiment being unchanged. Among the three cell preparations studied only one showed an uncertain positive reaction, the other two being negative. Therefore the blocking effect of the unconjugated antiserum may not necessarily demonstrate an immunological specificity but may be a non specific effect between goat serum and human gamma globulin. In the present experimental system the use of unconjugated antiserum in an intermediate step, usually regarded as a reliable control of specificity, is thus dubious.

When non specific attachment of human gamma globulin in the first step to cells from control rabbits is investigated with the indirect method, a test of specificity of its second step is necessary. This was already partly done using conjugated goat anti guinea pig globulin (see p 32[illegible]). Further controls were obtained by blocking the conjugated goat anti human globulin with human gamma globulin. The blocked conjugate was now pretreated with concentrated normal rabbit serum and used for the final step, the first step being treatment with human gamma globulin in saline. Cell preparations from two untreated controls and three sero-negative rabbits showed no fluorescence after this treatment—when blocking was made with saline all showed a strong generalized fluorescence similar to that shown in Fig. 2.

Finally the specificity of the reaction found in one rabbit was analysed. Its cells gave a positive fluorescence after a first step incubation with concentrated normal rabbit serum and a final step with conjugated goat anti human globulin pretreated with concentrated normal rabbit serum (see p 326). Blocking of the conjugated antiserum globulin with an excess of human gamma globulin gave negative reaction

Also when conjugated goat anti-guinea-pig globulin was used in the final step, a negative result was obtained Thus, also this reaction is apparently specific

4 Further Technical Studies

In order to study different test systems, as outlined above, it was necessary to keep slides with cell films frozen for a considerable time The effect of the freezing and of storing below —20° C on the outcome of specific and non-specific fluorescence reaction was explicitly studied

Two series of slides from rabbits injected with Freund's complete adjuvant were studied for non-specific generalized fluorescence with the indirect method: a first step with human gamma globulin in saline and a final step with conjugated goat anti human globulin in saline Slides were studied before freezing, after one day of storage in deep-freeze, and after one and two months of storage Representative colour pictures were taken on each occasion The non specific fluorescence did not change

Slides from 11 rabbits immunized with human gamma globulin and showing specific positive cells were also studied with the indirect method human gamma globulin preincubated with concentrated normal rabbit serum as a first step, and conjugated goat anti-human globulin preincubated with rabbit serum concentrate as the final step Any differences were not found before and after freezing, nor after storage for up to one year

Thus, freezing and storage do not influence the result of the fluorescence technique

For preincubation of the human gamma globulin with normal rabbit serum a one per cent solution of human gamma globulin in a concentrated pool of normal rabbit sera (cf "Methods") was used In some cases, to save concentrated rabbit serum a 0 25 per cent solution of human gamma globulin in unconcentrated pooled normal rabbit serum was used and the conjugated goat antiserum globulin was now also preincubated with an equal volume of unconcentrated normal rabbit serum Comparison between these two modifications usually showed no difference

DISCUSSION

In the present paper, possibilities of demonstrating with immunofluorescence methods antibody-containing lymphoid cells taken from rabbits immunized with human gamma globulin is analysed The direct immunofluorescence method used is not sufficently sensitive for this purpose but the indirect method can be used The observations are complicated by a number of non-specific reactions The observable conjugated goat anti-human globulin may be attached to the cells in various ways

a) A specific immunologic reaction obtained by a coupling of the fluorescent antibody to human gamma globulin specifically combined with those lymphoid cells that contain antibodies against human gamma globulin This is the desired reaction

b) Fluorescent components possibly although not necessarily antibody, may become attached to the cells without any interaction with human gamma globulin This phenomenon is a well known complication in immunofluorescence studies and has been attacked in different ways for instance by various treatments of the conjugate (cf Introduction) It was observed with different strengths in cell preparations from different rabbits The presence of normal rabbit serum in the first step strengthens this type of fluorescence (cf *Beutner* 1961 also *Mayersbach & Schubert* 1960)

c) The lymphoid cells may contain human gamma globulin given as antigen and therefore directly react with the conjugated goat anti human globulin This was seen only once (see p 326)

d) A non specific coupling of human gamma globulin to rabbit cells is a consistent and pronounced phenomenon At indirect immunofluorescence this phenomenon will cause a generalized fluorescence due to the non specific reaction between the unconjugated middle layer and the cells As mentioned in the introduction little interest has been paid to this subject in the literature This attachment of human gamma globulin to the rabbit cells could be the result of a non specific protein absorption, or it could represent an immunologic reaction between the human gamma globulin and antigens present in the rabbit cells These antigens may cross react with some unknown antigen that has resulted in an antibody formation in man The present author finds the former explanation more likely

e) Theoretically the goat anti human or anti guinea pig globulin can react immunologically with rabbit protein on the cell surface and produce fluorescent staining Either the conjugated goat antisera globulins used may be contaminated by traces of anti rabbit serum or rabbit proteins (*e g* gamma globulin) may have antigens common with human and guinea pig serum globulin

Schematic illustrations of these five alternatives are given in Fig 3

Two of these reactions (b d) can be blocked by pretreating the human gamma globulin and the conjugated goat antiserum globulin with normal rabbit serum A component common to serum and lymphoid cells is thus reasonably responsible for this non specific binding Therefore a trial was made to block the reactions by pre incubation with rabbit gamma globulin A solution of 36 mg/ml in saline was used because it was supposed to correspond to the gamma globulin concentration found in a concentrated serum (normal gamma globulin concentration in rabbits is given by *Andersen & Bjorneboe* 1964) This was studied on cells from one normal rabbit and four rabbits immunized with human gamma globulin in previous studi

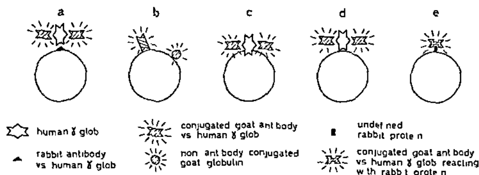


Fig 3

Schematic drawings illustrating five possible methods of staining with fluorescein conjugated goat antiserum globulin with lymphoid cells at indirect method *a* specific fluorescence, caused by the presence in the cells of antibodies against human gamma globulin b non specific fluorescence, caused by direct coupling between cell and fluorescent component in goat antiserum globulin c fluorescence caused by the presence of human gamma globulin as an antigen in the cell d fluorescence, caused by non specific absorption of human gamma globulin to the cell, the final step being specific e fluorescence caused by immunologic reaction between conjugated goat antiserum globulin and rabbit protein Further explanation in text

found to show a specific positive reaction No difference could be found between slides made with rabbit gamma globulin and those made with normal rabbit serum as preincubation medium Thus, the gamma globulin of normal rabbit serum has the same capacity of blocking non specific reactions as whole serum, it cannot be excluded that other serum components have the same effect The hypothesis that the non-specific reactions were due to non immunologic coupling of gamma globulins from the different species used seems reasonable

SUMMARY

Non specific staining reactions at immunofluorescence were studied using lymphoid cells from rabbits immunized with human gamma globulin and from various control rabbits

When the direct method was used, conjugated human gamma globulin formed a non-specific attachment with lymphoid cells, irrespective of the source of origin *This did not occur* when *the human gamma glob* ulin was pretreated with normal rabbit serum This method, however was too unsensitive to permit visualization of antibody-containing cells from immunized rabbits

With the indirect method, including treatment of the cells with unconjugated human gamma globulin followed by fluorescein con jugated goat anti-human globulin, a generalized non specific fluorescence occurred This was mainly due to a non specific attachment of the unconjugated human gamma globulin to the cells This interaction in the middle layer was eliminated when the human gamma globulin was pretreated with normal rabbit serum Also the conjugate

of the second step could react non specifically with the cells. This reaction did not occur when also the conjugate was treated with normal rabbit serum. The pretreatment of both steps, eliminating both types of non specific fluorescence, did not prevent the specific fluorescence obtained by reaction of human gamma globulin with antibody containing lymphoid cells from immunized rabbits.

Rabbit gamma globulin can be substituted for rabbit serum at pretreatments. This suggests by way of explanation of the non specific staining an interaction between cellular rabbit gamma globulin and human, perhaps also goat gamma globulin added to the cells.

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Acta path et microbiol scandinav 69 332 342, 1967

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INDUCTION OF THYROIDITIS IN GUINEA PIGS BY SERUM FROM RABBITS IMMUNIZED WITH GUINEA PIG THYROGLOBULIN

By

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Received 15 viii 66

Ever since the demonstration of precipitating antibodies against human thyroglobulin in serum of patients suffering from Hashimoto's disease (21) and the production of thyroglobulin antibodies and thyroiditis by immunization with homologous thyroid extracts in experimental animals (28, 24), the pathogenic capacity of thyroid antibodies in serum has been questioned

The problem has been approached in several ways

1 Transfer of serum from individuals with thyroiditis to normal recipients *Roitt & Doniach* (20) transferred serum from patients with high levels of thyroid antibodies to monkeys However no functional or morphological alterations of the monkey s thyroid could be detected *Rose Kite & Doebbler* (23) failed to transfer thyroiditis by repeated injections of rabbit anti rabbit thyroid immune serum into healthy rabbits
2 Studies on the offspring of females with thyroid antibodies in serum Although thyroid antibodies cross the placenta barrier no functional or histological damage has been observed in the thyroid of newborn children (19) rabbits (2) or guinea pigs (25)
3 *Segregation of thyroiditis and thyroid antibodies Thyroiditis may be produced* in guinea pigs without induction of serum antibodies (17) On the other hand thyroid antibodies may be induced without histological alterations of the gland (23)
4 Lack of ... thyroiditis The course a ... closely related to delayed ... n serum thyroid antibod
5 Transfer of thyroid immune serum produced by heterologous immunization *Roitt Jones & Doniach* (22) injected serum from rabbits immunized with rat thyroglobulin into rats No damage of the recipients thyroid was observed when serum was given alone Animals previously exposed to ionizing radiation or Freund adjuvants however revealed thyroiditis within a few days after intra peritoneal injections of thyroid immune serum The histological alterations apparently consisted of infiltration of the thyroid by mon nuclear cells

The present investigation followed the last approach As recipients guinea pigs were employed with the rabbit as the antibody producing species

MATERIALS AND METHODS

Animals Randomly bred white female guinea pigs, strain AC, with body weights of 250-350 grams were obtained from the National Institute of Public Health, Oslo. Randomly bred rabbits initially weighing 2000-3000 grams were employed for production of antiserum.

Antigens Thyroid glands of CO_2 killed and desanguinated guinea pigs were excised, trimmed of fat and cut into small pieces. After addition of 2 volumes saline (w/v) the mixture was homogenized at 0° C and stirred at 4° C over night. After centrifugation (4000 × g × 30 minutes) the supernatant was used for separation of thyroglobulin. Thyroglobulin was prepared by $(NH_4)_2SO_4$ fractionation between 30 and 50 per cent saturation with subsequent dialysis against running water for 24 hours, distilled water for 24 hours and saline for 24 hours. The thyroglobulin preparation was stored at −20° C.

Extracts of guinea pig kidney, liver and spleen were prepared in an identical manner. After centrifugation (4000 × g × 30 minutes) the supernatant was employed for immunization and gel precipitation studies.

Antisera Rabbits were immunized with guinea pig thyroglobulin or guinea pig kidney extracts. Five mg of antigen per ml of saline was mixed carefully with equal volumes of Difco's Freund's complete adjuvants. 0.0 ml of the mixture was injected as superficially as possible in all 4 foot pads once a week for 4 weeks. The rabbits were bled 3 times during the second week after the last injection and the serum obtained was pooled. Two months later the animals were boostered by one injection into each foot pad and bled a second time. Guinea pigs were immunized in a similar way with rabbit serum by injection of 0.025 ml into each of the foot pads.

Precipitation in gel The method of *Ouchterlony* (18) as described by *Eriksen* (3) was employed. Constituents of the gel were 0.03 M barbital buffer at pH 8.6, 0.003 per cent methyl orange, 1 per cent Difco Special Agar Noble and 0.1 per cent phenol.

Hemagglutination The indirect hemagglutination procedure of *Boyden* (1) was employed. Human erythrocytes were formalinized and sensitized with guinea pig thyroglobulin as previously described (5, 6, 7) with employment of normal guinea pig serum instead of normal rabbit serum as stabilizer. The concentration of antigen used for sensitization was 0.5 mg per ml against 1.2 × 10^8 cells per ml. Tanned uncoated erythrocytes were used for control of specificity. The sera were screened in dilutions 1:5, 1:25, 1:250, 1:2500 etc. with two-fold titration intervals when a more precise titer was desired.

[illegible] ermined by the biuret method

[illegible] ther killed and desanguinated

[illegible] Tissue sections were either stained with hematoxylin and eosin or by the May-Grunwald and Giemsa technique.

RESULTS

Specificity of Rabbit Antisera

Serum of rabbits immunized with guinea pig thyroglobulin or kidney extract appeared to react with guinea pig serum in Ouchterlony plates. Consequently, rabbit immune serum was absorbed with guinea pig serum. Absorption was performed at 4° C in several steps by using guinea pig serum:rabbit antiserum ratios of 1:50, 1:10 and 1:2. The anti-guinea pig thyroglobulin serum did then not react with guinea pig plasma as shown in Fig. 1. Rabbit immune serum against guinea pig kidney had to be absorbed 1:50, 1:10, 1:2, 1:2 and 1:2 before all antibodies against guinea pig plasma were removed as tested by precipitation in gel (Fig. 1). Thyroglobulin antiserum was found to react with a single line against preparations of guinea pig thyroglobulin as well as whole thyroid extracts but did not react with extracts

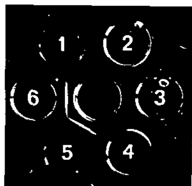


Fig 1

thyroid extract (5 mg protein per ml) 6 Guinea pig thyroglobulin (5 mg per ml)

guinea pig liver, spleen or kidney On the other hand, immune serum against guinea pig kidney gave rise to 2 lines against kidney extracts but did not react with thyroglobulin or thyroid extracts

The absorbed thyroglobulin immune serum gave rise to a hemagglutination titer of 1 100 000 by cells coated with guinea pig thyroglobulin

Histologic Alterations in the Thyroid Gland of Guinea Pigs Following Intraperitoneal Application of Rabbit Immune Serum Against Guinea Pig Thyroglobulin

As shown in Fig 3 a as compared to Fig 2, remarkable histological changes were found in the thyroid gland of guinea pigs injected 24 hours previously with thyroglobulin immune serum In histological sections confluent infiltrations of polymorphonuclear leukocytes were observed The granulocytes were mainly located interstitially but rupture of follicles with streaming of granulocytes into the colloid was frequently seen (Fig 3 b) Proliferation of histiocytes seemed not to be predominant at that time and occasionally only, lymphocytes were observed Blood vessels appeared normal The granulocytes revealed rod shaped or segmented nuclei with 2–3 globules, and their cytoplasmic granules were stained slightly eosinophilic by hematoxylin and eosin (Fig 3 b) and red by the May-Grünwald and Giemsa staining (Fig 3 c) Blood smears stained by the May Grunwald and Giemsa technique were found to contain less than 1 per cent of such cells while approximately 50 per cent of the leukocytes consisted of granulocytes with hypersegmented nuclei and with small and poorly stained cytoplasmic granules (Fig 4) These observations would seem

TABLE 1

Histological and Serological Findings at the Time of Sacrifice in Guinea Pigs Receiving Intraperitoneal Injections of Rabbit Anti-Guinea Pig Immune Sera

Animal no	Antiserum injected	Volume (ml) injected	Time of sacrifice	Hemagglutination titer of thyroglobulin antibody: At the time of sacrifice	Hemagglutination titer of thyroglobulin antibody: After absorption with guinea pig anti-rabbit serum	Severity of histological lesion in the thyroid
Y 1	anti guinea pig thyroglobulin	4	24 hours	1:20 000	—*	+++
Y 2			"	1:10 000	—	++
Y 3			48 hours	1:5 000	—	0
Y 4	"	"		1:20 000	—	+
Y 5			5 days	1:10 000	—	++
Y 6				1:10 000	—	+++
Y 7			10 days	1:5 000	<1:25	+
Y 8	"		"	<1:5	—	0
Y 9			20 days	1:25	<1:5	0
Y 10				1:1 000	<1:25	+
Y 11	anti guinea pig kidney extract		24 hours	<1:5	—	0
AB 17	"		"	<1:5	—	0
Y 12			5 days	<1:5	—	0
AB 18	"		"	<1:5	—	0
Y 13			10 days	<1:5	—	0
AB 19			"	<1:5	—	0
Y 14		"	20 days	<1:5	—	0
AB 20			"	<1:5	—	0

* — not done

Graded according to *Jones & Roitt* (10): + — separate foci of inflammatory cells; ++ — confluent infiltration of inflammatory cells; +++ — confluent infiltration of inflammatory cells with destruction of follicular architecture

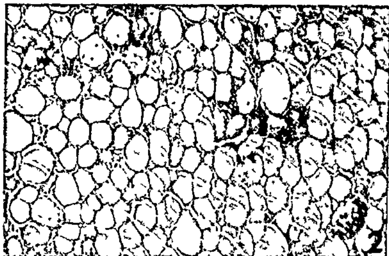


Fig 2
Normal appearance of a guinea pig thyroid (animal Y-11), (hematoxylin and eosin × 70)

to indicate that the granulocytes found in the thyroid of guinea pigs receiving thyroglobulin immune serum are eosinophils

Five days after the injection of immune serum, there were still confluent infiltration of granulocytes, but the dominant cell type at that time was the histiocyte (Fig 5 a and b) There was a moderate increase in the number of lymphocytes

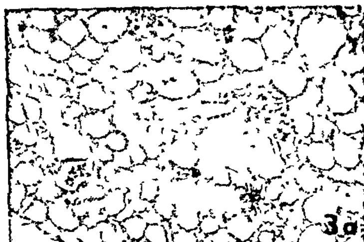
Ten or 20 days after the injection of immune serum, the histologic alterations of the thyroid had regressed and changed in character Only 2 out of 4 animals showed a mild degree of thyroiditis (Table 1) The thyroid of the other 2 animals appeared normal Although granulocytes still could be observed, they were mainly located intrafollicularly Small groups of lymphocytes and slight fibrosis were now seen in the interfollicular spaces (Fig 6)

None of the animals injected with rabbit immune serum against guinea pig kidney revealed histological changes of their thyroid (Fig 2) and no pathological changes could be observed in the liver or kidney of any animal

Most of the animals receiving thyroglobulin immune serum on the day of sacrifice appeared to have large quantities of circulating rabbit

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Fig 3
Thyroiditis (grade ++) in a guinea pig 24 hours after injection of rabbit anti guinea pig thyroglobulin (animal Y 1) a) Interstitial and follicular invasion of (hematoxylin and eosin × 70) b) predominantly polymorphonuclear leukocytes (hematoxylin and eosin × 175) c) which are stained red by May Grunwald and Giemsa (May Grunwald and Giemsa × 175)


3a


3b


3c

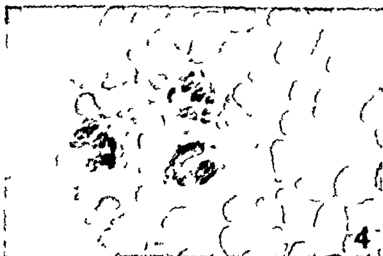


Fig 4

A blood smear stained by May Grunwald and Giemsa showing 1 cell with a nucleus consisting of 2 globules and with a cytoplasm rich in red coloured granules Less than 1 per cent of the leukocytes consisted of such cells The other 2 cells found in a frequency of about 50 per cent have a hypersegmented nucleus with few and poorly stained cytoplasmic granules (May Grunwald and Giemsa × 1080)

antibodies against guinea pig thyroglobulin (Table 1) Even in animals sacrificed 10 or 20 days after injection of rabbit immune serum all the thyroglobulin antibodies were removed by absorption with guinea pig immune serum against rabbit serum (Table 1) Apparently all thyro globulin antibodies left in serum were of rabbit origin

DISCUSSION

The present study has shown that heterologous immune serum against thyroglobulin may affect the thyroid of healthy guinea pigs These results are at variance with those of *Roitt, Jones & Doniach* (22) who observed effects of heterologous immune serum only on the thyroid of rats pretreated with ionizing radiation or Freund adjuvants Pretreated animals revealed a type of thyroiditis similar to the inflammation which appears after active immunization with mixtures of thyroid extracts

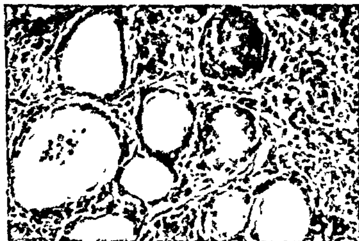
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Figs 5–6

Fig 5 Thyroiditis (grade +++) in a guinea pig 5 days after receiving rabbit anti guinea pig thyroglobulin (animal Y–6) a) Confluent infiltration with destruction of follicular architecture (hematoxylin and eosin × 70) b) by mononuclear cells predominantly of the histiocytic type some follicles still contain granulocytes (hematoxylin and eosin × 175)

Fig 6 Thyroid of a guinea pig 20 days after receiving rabbit anti guinea pig thyro globulin showing small interstitial infiltrations of lymphocytes and granulo cytes intrafollicularly (animal Y 10) (hematoxylin and eosin × 70)


a


b


6

or thyroglobulin in Freund adjuvants The last type of thyroiditis may in early stages occasionally reveal infiltration of granulocytes (10, 13, 26, 29), but the dominant inflammatory cell types are lymphocytes and histiocytes

Although the present type of thyroiditis contains the same elements, the quantitative differences are remarkable Dominant in the present inflammation is the rapid infiltration of granulocytes which seems to be eosinophils, while the delayed aggregation of lymphocytes is little pronounced

The mechanism behind this reaction at present remains unknown Since the reaction is limited to the thyroid and specific to rabbit immune serum against guinea pig thyroglobulin, the factor responsible most likely is a thyroglobulin antibody Serological studies support this hypothesis (Fig 1)

Since *Litt* (14) has shown that eosinophils may be attracted by antigen-antibody complexes, the explanation may be that thyroglobulin antibody complexes attract the granulocytes which seem to be eosinophils As is the case with classical experimental thyroiditis, most of the inflammatory cells are located interstitially, although the colloid of several follicles may be invaded by inflammatory cells This aggregation in the interfollicular spaces may be due to the presence of small quantities of thyroglobulin in the interstitial spaces or, the follicle wall may represent a barrier which is difficult for the leukocytes to penetrate Whether the streaming of cells into the colloid has relation to colloidophagy (8), at present remains unknown

The delayed infiltration of lymphocytes in the present type of thyroiditis could be a part of an immunological reaction against foreign antibodies in parallel with the Masugi type of nephritis (12) However, since animals with the highest titers of rabbit thyroglobulin antibodies revealed infiltration of mononuclear cells, while animals in which these antibodies had been remarkably reduced did not show infiltration of such cells in their thyroid, the delayed aggregation of lymphocytes may not be a part of an immunological response against the foreign antibodies

Besides the Masugi nephritis (15), which involves the delicate vascular structures of the glomerulus previous studies have indicated that serum antibodies only may affect parenchymatous tissues when animals are given certain kinds of pretreatment (22) or when it is applied locally (9) The present study shows that heterologous serum antibodies in healthy recipients through the circulation may reach extravasally located antigens of parenchymatous tissues and initiate an inflammatory reaction

SUMMARY

Histological changes in the thyroid of guinea pigs following intraperitoneal injection of rabbit anti guinea pig thyroglobulin immune serum

are described Within 24 hours thyroiditis develops with a massive infiltration of polymorphonuclear leukocytes which seem to be eosinophils The acute stage is succeeded by proliferation of mononuclear cells, mainly histiocytes, and by a less pronounced delayed aggregation of lymphocytes

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INDUCTION OF THYROIDITIS IN GUINEA PIGS BY SERUM FROM GUINEA PIGS IMMUNIZED WITH GUINEA PIG THYROGLOBULIN

By

TORE GODAL and ROLF KÅRESEN

Received 22.viii.66

In a previous report (2) development of thyroiditis in guinea pigs subsequent to intraperitoneal injections of rabbit anti-guinea pig thyroglobulin immune serum was described. In order to achieve information about the biological importance of this thyroiditis, the present study, with employment of homologous instead of heterologous immune serum was undertaken.

MATERIALS AND METHODS

Animals. Randomly bred, white, female guinea pigs, strain AG, with body weights of 250–350 grams were obtained from the National Institute of Public Health, Oslo.

The preparation of antigens and the serological and histological methods were the same as previously reported (2).

Antisera. Guinea pigs were immunized with homologous thyroglobulin mixed with Freund adjuvants or with Freund adjuvants only. Five mg per ml of thyroglobulin or 0.15 M saline were carefully mixed with equal volumes of Difco's Freund complete adjuvants. 0.02–0.03 ml of the mixtures were injected into each foot pad once a week for 4 weeks. The animals were bled by cardiac puncture 3 times during the second week after the last injection.

Four types of antiserum were prepared.

1. *AI or B.* This type of anti[illegible]
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2. [illegible] animals were again bled by cardiac puncture after injections once a week for 4 weeks. Sera with thyroglobulin antibody titers of ≥ 1:1000 were included in this type of antiserum. Several of the individual antisera failed to give a positive precipitation reaction. Serum AoII gave rise to a titer of 1:2500 with thyroglobulin sensitized red cells.
3. *AoI.* Sera from animals failing to respond to booster injections of thyroglobulin with titers ≤ 1:50 were included in this type of antiserum.
4. *C.* Antiserum C was prepared from animals immunized with Freund adjuvants only. Antiserum C failed to agglutinate thyroglobulin sensitized red cells (<1:5).

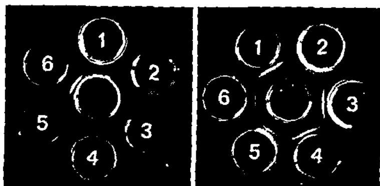


Fig 1 *Fig 2*

Fig 1 Precipitation in gel by guinea pig immune sera and guinea pig tissue antigens Central well Guinea pig anti guinea pig thyroglobulin (antiserum B A_1 or A H) 1 4 Guinea pig anti Freund adjuvants (antiserum C) 2 Guinea pig liver extract (5 mg protein per ml) 3 Guinea pig kidney extract (5 mg protein per ml) 5 Guinea pig thyroid extract (5 mg protein per ml) 6 Guinea pig thyroglobulin (5 mg per ml)

Fig 2 Precipitation in gel by heterologous and homologous antisera against guinea pig thyroglobulin and guinea pig thyroglobulin Central well Guinea pig *thyroglobulin (5 mg per ml) 1 4 Guinea pig anti guinea pig thyroglobulin* type B (or A_1) 2 5 Guinea pig anti guinea pig thyroglobulin type A-H 3 6 Rabbit anti guinea pig thyroglobulin absorbed with guinea pig serum (diluted 1 5)

RESULTS

Specificity of Antisera

As demonstrated in Fig 1 thyroglobulin antisera (A_1 B or A-H) gave rise to an identity reaction between thyroglobulin and an unfractionated thyroid extract with a single precipitin band No reaction was observed against extracts of guinea pig kidney or liver Furthermore the various types of antisera between themselves also revealed an identity reaction against thyroglobulin (Fig 2) This identity reaction was extended to include rabbit anti guinea pig thyroglobulin immune serum (Fig 2) which previously has been employed for induction of thyroiditis (2) Serum C failed to react with any of the guinea pig tissue extracts (Fig 1)

Histological Changes in the Thyroid of Guinea Pigs Following Intraperitoneal Injection of Guinea Pig Anti Guinea Pig Thyroglobulin Immune Serum

The histological alterations present at various intervals after a single intraperitoneal injection of 16–25 ml of homologous thyroglobulin immune serum are summarized in Table 1 Twenty four hours after injections of antiserum A_1 or B confluent infiltrations of granulocytes throughout the thyroid were observed (Fig 3 a) By the May Grunwald and Giemsa technique the cytoplasmic granules of the polymorphonuclear leukocytes were stained red and their nuclei were rod shaped

TABLE 1

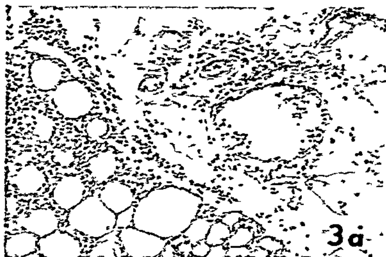
Histological and Serological Findings at the Time of Sacrifice in Guinea Pigs Receiving Intraperitoneal Injections of Guinea Pig Anti Guinea Pig Thyroglobulin or Guinea Pig Anti Freund Adjuvants Immune Serum

Animal no	Antiserum injected: Type*	Antiserum injected: Volume (ml)	Antiserum injected: Hemagglutination titer of thyroglobulin antibody	Time of sacrifice	Hemagglutination titer of thyroglobulin antibody at sacrifice	Histological findings in the thyroid: Severity of lesion	Histological findings in the thyroid: Infiltration of granulocytes
Z–7	A1	16	1:1000	24 hours	1:250	+++§	interstitial and capsular"
Z–8	"	16	1:1000	24 hours	1:250	++	
AB–5	B	20	1:500	24 hours	1:100	++	"
Z–9	A1	16	1:1000	5 days	—†	+	
Z 10	"	20	1:1000	5 days	1:250	+	"
AB 6	B	20	1:500	10 days	—	0	0
AB 7	D	20	1:500	20 days	1:5	0	0
AB 4	A1	25	1:1000	30 days	1:5	0	0
AB 1		25	1:1000	30 days	<1:5	0	0
AB		25	1:1000	30 days	<1:5	0	0
AB 8	A-II	20	1:2500	24 hours	1:250	+	0
AB 9		20	1:2500	5 days	1:500	+	"capsular"
AB 10	"	20	1:2500	10 days	1:250	+	
AB 14	A-I	20	<1:25	24 hours	<1:5	+	0
AB 15		20	1:25	5 days	1:5	+	0
AB 16		20	<1:25	10 days	<1:5	0	0
AB 11	C	20	<1:5	24 hours	<1:5	+	0
AB 12	C	20	<1:5	5 days	<1:5	0	0
AB 13	C	20	<1:5	10 days	<1:5	0	0

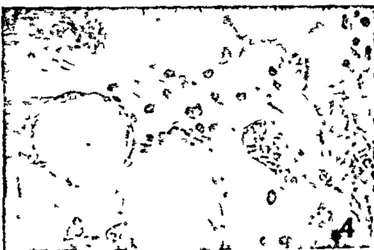
* A1 or B Guinea pig anti guinea pig thyroglobulin immune serum obtained after 1 month of immunization. A-II Thyroglobulin immune serum from guinea pigs of group A responding to booster injections 4 months after the first period of immunization. A-I Serum from animals of group A which failed to respond to thyroglobulin by booster injections. C Anti Freund adjuvants immune serum.

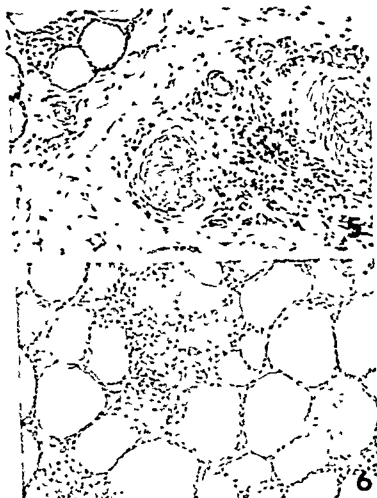
§ graded according to *Jones & Roitt* (6) + — separate foci of inflammatory cells, ++ — confluent infiltration of inflammatory cells.

† — not done


3a


3b


4



Figs 5 6

Fig 5 Thyroiditis (grade +) in a guinea pig 5 days after injection of guinea pig anti guinea pig thyroglobulin (antiserum A_1 animal Z 10) showing aggregation of granulocytes around vessels adjacent to the thyroid ("capsular" infiltration) (May Grunwald and Giemsa × 70

Fig 6 Thyroiditis (grade +) in a guinea pig 21 hours after injection of guinea pig anti Freund adjuvants (animal AB 11 showing an inflammatory focus consisting of lymphocytes and histiocytes (hematoxylin and eosin × 70)

Figs 3 4

Fig 3 Thyroiditis (grade ++) in a guin a p s 24 h urs after injection of guinea pig anti guinea pig thyrogl bulin (r m B animal AB 5) a) Interstitial and "capsular" infiltrati n h r at xylin and eosin × 70) b) by predominantly polymorphonuclear ... s with a hypo segmented nucleus and a cytoplasm rich in granu ... t r l by the May Grunwald and Giemsa staining (May Grünwal ... m a × 175)

Fig 4 Thyroiditis (grade +) in a gu ... s days after injection of guinea p anti guinea pig thyroglobulin ... r m A_1 animal Z 9) showing stitial infiltration of granul ... May Grünwald and Giemsa × 17

or consisted of a few globules (Fig 3 b) On this basis, these cells are *suspect of being eosinophils* (2) *Exceptionally only*, inflammatory cells were observed intrafollicularly

Five days after injection of thyroglobulin immune serum A_1 or B, the number of granulocytes had decreased without any marked increase of histiocytic elements or lymphocytes (Fig 4) The aggregation of granulocytes adjacent to the thyroid was at that time remarkable ("capsular infiltration") No animals sacrificed 10 50 days after injection of homologous thyroglobulin immune serum revealed any sign of thyroiditis

Animals receiving thyroglobulin antiserum of type A-H showed only a mild degree of thyroiditis after 24 hours Small infiltrations of histiocytes and lymphocytes could be observed But these changes were not significant since animals receiving homologous anti-Freund adjuvants immune serum (antiserum type C) may show such changes (Fig 6) Furthermore, similar histological changes could be observed in the thyroid *of animals receiving serum of type* A_2L (Table 1)

On the other hand, animals sacrificed 5 or 10 days subsequent to injections of serum A-H in addition to non specific changes revealed aggregation of granulocytes adjacent to the thyroid (see Fig 5) No animal receiving immune serum against Freund adjuvants only (antiserum type C) or against thyroglobulin with low levels of thyroglobulin antibodies (antiserum type A-L) revealed any infiltration of granulocytes in or adjacent to the thyroid gland Liver and kidneys of all animals appeared normal

DISCUSSION

The present study shows that guinea pig anti guinea pig thyroglobulin immune serum may initiate thyroiditis in guinea pigs In parallel with thyroiditis produced by heterologous immune serum (2) the predominant histological feature was a rapid infiltration of granulocytes apparently eosinophils

In contrast to heterologous immune serum, homologous thyroglobulin immune serum did not produce any significant intrafollicular aggregation of granulocytes any remarkable proliferation of histiocytes or a delayed infiltration of lymphocytes Since the quantities of heterologous thyroglobulin antibody employed for injection, as measured by the method of indirect hemagglutination, were 10–70 times that of homologous antibodies, the histologic changes only observed after injections of heterologous immune serum may be explained on a quantitative basis By injection of heterologous thyroglobulin immune serum toxic antigen antibody complexes (at moderate antigen excess, (5)) may be established intrafollicularly, while injections of smaller antibody quantities only produce such complexes in the interfollicular

spaces due to interfollicular presence of small amounts of thyroglobulin

On the other hand, there may be qualitative differences between heterologous and homologous thyroglobulin immune serum. Although only a single precipitin band could be observed against thyroid tissue in Ouchterlony plates, heterologous thyroglobulin immune serum may contain antibodies to other components of the thyroid which are not present in homologous thyroglobulin immune serum. Finally, since antibodies with the highest affinity to the antigen (7) produced in the same species may be removed from the serum by the thyroid gland of the immunized animal, heterologous immune serum may contain antibodies of higher affinity to the antigen. In this way, a heterologous condition may have features in common with an auto-immune reaction which are not present when auto-antibodies are transferred between animals of the same species

A mild degree of thyroiditis (+) was observed in animals receiving Freund adjuvants immune serum (antiserum C) or thyroglobulin immune serum with poor anti thyroglobulin response (antiserum A-I). The histologic alterations found in these animals consisted of small infiltrates of mononuclear cells. Since immunization with Freund adjuvants may induce scattered infiltration of mononuclear cells in the thyroid gland (3, 8), the present findings indicate that this potential in parallel with other effects of Freund adjuvants (1), may be transferred by serum

On the other hand aggregation of granulocytes, presumably of the eosinophilic type, was found only in or adjacent to the thyroid of guinea pigs receiving large quantities of thyroglobulin antibody. This observation strengthens the hypothesis that thyroglobulin serum antibodies may be capable of inducing granulocytic infiltration in or around the thyroid gland

Apparently the degree and type of granulocytic infiltration are not correlated to injected levels of hemagglutinating antibodies against thyroglobulin. One type of thyroglobulin immune serum (A, or B) prepared after 1 month of immunization gave rise to thyroglobulin antibody titers of 1 1000 or 1 500. This type of antiserum initiated an acute interstitial and "capsular" infiltration of granulocytes. The other type of high titered thyroglobulin immune serum (A-II thyroglobulin antibody titer of 1 2500) prepared subsequent to booster injections 4 months later initiated a delayed and less pronounced capsular aggregation of granulocytes. "Capsular" infiltration of eosinophils has previously been recognized in rabbits after immunization with thyroid extracts in Freund adjuvants (9

The two types of antisera rev[illegible] ... ality reactions in Ouchterlony plates but antiserum A-II ma[illegible] ... higher hemagglutination pr[illegible] cipitation ratio than antiserum ... A or B. Since guinea pig ant[illegible] bodies to guinea pig thyrogl[illegible] ... y consist of various typ[illegible] t

immune globulins (4), the explanation behind the lack of correlation between histological alterations and hemagglutinating antibodies may be that different types of immune globulins are involved

SUMMARY

Thyroiditis in guinea pigs, following intraperitoneal injection of guinea pig anti guinea pig thyroglobulin immune serum, is described The histological lesions are dominated by infiltration of granulocytes, apparently eosinophils Although the specificity of the reaction indicates that thyroglobulin antibodies may initiate such changes the severity of thyroiditis does not correlate with the amounts of thyroglobulin antibody injected as measured by the method of indirect hemagglutination

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Acta path. et microbiol. scandinav. 69: 351–356, 1967

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HYPERSEGMENTATION IN NEUTROPHILIC GRANULOCYTES

By

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Received 11.viii.66

Hypersegmentation of the nucleus of granulocytes has long been known to accompany megaloblastic anaemia, especially when there is vitamin B_{12} deficiency. The exact explanation of the phenomenon is not known.

The nuclear formula of neutrophils with respect to segmentation is generally believed to be represented by a bell-shaped curve with a maximum at about three segments (1, 9). There has been some discussion regarding the occurrence of hypersegmentation in other conditions than megaloblastic anaemia. Thus, *Edwin et al.* (3) state that they "believe that hypersegmented granulocytes may be found in several other conditions than hypovitaminosis B_{12}." The matter is complicated by the changes in vitamin B_{12} metabolism which occur in several haematological conditions. These changes have recently been reviewed by *Begemann et al.* (2).

It was thought that a study of the segmentation of neutrophilic granulocytes in a clinical series might contribute to the understanding of the clinical significance of hypersegmentation.

MATERIAL AND METHODS

100 patients were randomly chosen among those who had been subjected to bone marrow examination during 1965. After exclusion of those patients whose B_{12} or folic acid metabolism was not certainly known, there remained 78 patients whose peripheral blood was investigated. The diagnoses in these cases are shown in Table 1. Additionally, 20 healthy control subjects, all nurses or hospital technicians, were studied. Of these healthy controls, 10 were females and 10 were males.

The total number of persons in this study is thus 98. Vitamin B_{12} deficiency was ruled out or revealed by serum B_{12} determinations (microbiological determination [illegible]

immersion with a 10 × ocular and 100 [illegible] objective. A differential count was performed, after which the neutrophilic granulocytes were inspected separately and classed according to their number of segments. When a suitable number of cells had been examined, the number of cells in e[illegible] was expressed as a percentage of the total neutrophil count.

TABLE 1

Diagnoses of all Subjects Included in the Study

B_{12} hypovitaminosis

7 Addisonian pernicious anaemia
1 Intestinal resection syndrome
1 Protein losing enteropathy

Iron deficiency

18 patients

Other erythrocytic disorders

1 Erythroid aplasia
1 Hereditary sphaerocytosis
5 Polycythaemia vera

Chronic myelogenous leukaemia

1 patient

Other haematological disorders

3 Idiopathic thrombocytopenia
1 Allergic purpura
1 Eosinophilia of unknown origin
1 Incipient myeloproliferative syndrome

Malignancy

1 Astrocytoma
1 Bronchial carcinoma
1 Pulmonary carcinoma
1 Ventricular carcinoma
1 Hepatic carcinoma
2 Hodgkin's disease
1 Metastatic carcinoma of unknown origin

Infection

1 Acute upper respiratory infection
1 Chronic pyelonephritis
1 Purulent arthritis
1 Staphylococcus aureus septicaemia
2 Subacute bacterial endocarditis
2 Tuberculosis of lymph nodes

Myeloma and macroglobulinaemia

2 Myelomatosis (Plasmocytoma)
1 Macroglobulinaemia

Other patients

1 Ankylosing spondylarthritis
2 Cerebral apoplexy
1 General arteriosclerosis
1 Coronary artery failure
3 Diabetes mellitus
1 Duodenal ulcer
1 Pleural adhesions
1 Ventricular polyposis
1 Portal hypertension
1 Renal arteriosclerosis with hypertension
1 Sarcoidosis
1 Sjogren's syndrome
1 Spondylarthrosis
1 Terminal ileitis
1 Thyrotoxicosis

20 Healthy subjects

RESULTS

The results are shown in Tables 2–4 As would be expected, B_{12} hypovitaminosis forms the group with the most marked tendency to hypersegmentation In this group, the greatest number of 6 segmented cells is seen, and some cells with 7 segments are also encountered In several other groups, however, in fact in all except the myeloma group and the healthy controls, occasional cases of hypersegmentation are observed But 7-segmented cells are seen only in the malignancy group and the infection group, although cases with 6-segmented cells are abundant

DISCUSSION

The cause of hypersegmentation is unknown If the phenomenon is interpreted as a sign of vitamin B_{12} deficiency then the question arises Do the present results indicate that there was, *after all*, vitamin B_{12} deficiency in those patients who had hypersegmented neutrophils but no other detectable signs of such a lack? We know from the investigations of *Killander & Larsson* (5) that low B_{12} values may be encoun

TABLE 2

Numbers of Cells Expressed as Percentages of Total Neutrophil Count

	No. of subjects	Number of nuclear segments* 1	2	3	4	5	6	7
B_{12} hypovitaminosis	9	0 7 2	0 11 3	8 33 26	32 49 41	5 40 20	0 20 7	0 3 <1
Iron deficiency	15	0 23 6	4 20 10	22 52 40	14 45 33	2 27 10	0 6 1	
Other erythrocytic disorders	7	1 19 7	4 19 10	18 47 35	20 43 33	5 15 12	0 4 2	
Chronic myelogenous leukaemia	1	43	5	20	20	10	2	
Other haematological disorders	6	1 60 13	4 21 12	18 51 31	10 40 33	2 20 10	0 10 3	
Malignancy	8	0 23 12	0 25 16	2 43 33	8 55 25	0 30 10	0 10 3	0 4 <1
Infection	8	1 78 21	3 24 14	14 54 34	6 41 24	0 20 6	0 8 1	0 1 <1
Myeloma and macroglobulinaemia	3	3 6 5	8 21 17	45 50 48	21 37 27	2 6 3		
Other patients	18	0 45 6	0 30 10	20 73 41	4 55 36	0 21 7	0 3 <1	
Healthy subjects	20	1 13 5	11 32 20	34 50 43	16 35 27	1 12 5		

* Upper row: Range. Lower row: Mean.

TABLE 3

Number of Subjects with Cells in Each Group

	Total No.	Number of nuclear segments 1	2	3	4	5	6	7
B_{12} hypovitaminosis	9	[illegible]	6	9	9	9	6	2
Iron deficiency	18	16	18	18	18	18	4	
Other erythrocytic disorders	7	7	7	7	7	7	5	
Chronic myelogenous leukaemia	1	1	1	1	1	1	1	
Other haematological disorders	6	6	6	6	6	6	2	
Malignancy	8	7	7	8	8	6	2	1
Infection	8	8	8	8	8	6	2	1
Myeloma and macroglobulinaemia	3	3	3	3	3	3		
Other patients	18	15	18	[illegible]	19	14	5	
Healthy subjects	20	20	20	20	20	19		

tered in myelomatosis. In the present study, however, this does not present any problem, as the myeloma group did not show cells with more than five nuclear segments. *Mollin & Ross* (7) found high serum B_{12} values in polycythaemia, whereas *Be[illegible]mann et al.* (2) observed low values. The latter also found low serum B_{12} values in lymphadenosis and in macroglobulinaemia. All these investigators found high serum B_{12} values in patients with chronic myelogenous leukaemia. In the present study, the patient with chronic myelogenous leukaemia had some hy-

TABLE 4

Diagnosis of the Patients Who Had Cells with more than Five Nuclear Segments

	6 segments	7 segments
B_{12} hypovitaminosis	5 Addisonian p a 1 intestinal resection syndrome	2 Addisonian p a
Iron deficiency	4 patients	
Other erythrocytic disorders	1 erythroid aplasia 1 cong spherocytosis 3 polycythaemia vera	
Chronic myelogenous leukaemia	1 patient	
Other haematological disorders	2 idiopathic thrombocytopenia	
Malignancy	1 bronchial carcinoma 1 hepatic carcinoma	1 hepatic carcinoma
Infection	1 staph septicaemia 1 chronic pyelonephritis	1 chronic pyelonephritis
Other patients	1 ankylosing spondyl arthritis 1 cerebral apoplexy 1 duodenal ulcer 1 renal arteriosclerotic hypertension 1 sarcoidosis	

persegmentation, no abnormality of her B_{12} metabolism was detected This matter thus needs further clarification

It is probable that B_{12} hypovitaminosis might exist on the cellular level with a normal B_{12} absorption from the intestine if the B_{12} requirements are very high, as may be the case in rapid cellular proliferation, such as occurs in leukaemia, polycythaemia and carcinoma On the other hand, the high serum B_{12} values observed by several authors in chronic myelogenous leukaemia and in polycythaemia might signify inability of the diseased cells to utilize available B_{12}, owing, for instance to interference by the disease process with the B_{12}-binding capacity of these cells Views on the same lines have been expressed by *Mollin & Ross* (7)

Hypersegmentation should not be regarded as a step in the ageing of granulocytes, for these cells are usually destroyed through nuclear pyknosis without hypersegmentation, as has been shown both *in vivo* and *in vitro* (4, 6) It seems that hypersegmentation is due to a disturbance of the normal metabolic processes in the nucleus This disturbance may be caused by ineffective utilization of B_{12}, as suggested above or by some unknown factor independent of vitamin B_{12}

Schullen (10) regards hypersegmented granulocytes as a more ma-

ture form of the giant metamyelocytes or giant stab cells which are often seen in pernicious anaemia. Of the patients in the present study, only those with Addisonian pernicious anaemia had such giant cells in the bone marrow. *Müller* (8) studied a patient who had hypersegmented neutrophils during pregnancy in connection with iron deficiency and severe pyelonephritis. He did not see giant metamyelocytes or stab cells in his patient, although the hypersegmented neutrophils were unusually large.

Thus, there is a good deal of support for the assumption of *Edwin et al.* (3) that hypersegmented granulocytes would be found in other conditions than hypovitaminosis B_{12}.

Clinically, the matter is less complicated. Neutrophilic granulocytes with hypersegmented nuclei may obviously usually be regarded as a sign of B_{12} hypovitaminosis. Since hypersegmentation is also seen in other conditions, this phenomenon cannot be interpreted as a sign of vitamin B_{12} deficiency if other signs are lacking. In conjunction with other signs, however, hypersegmentation corroborates a diagnosis of vitamin B_{12} deficiency.

SUMMARY

The peripheral blood of 78 patients and 20 healthy subjects was examined with regard to nuclear segmentation of neutrophilic granulocytes. The greatest number of cells with 6 or more segments was found in hypovitaminosis B_{12}. In all other groups except the myeloma group and the controls, however, cases of hypersegmentation were also encountered.

The cause of hypersegmentation is discussed, with special reference to a possible undetectable B_{12} deficiency. Clinically, it is noted that hypersegmentation alone cannot be regarded as a sign of hypovitaminosis B_{12}, but is a useful adjunct to other signs of this deficiency.

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Acta path. et microbiol. scandinav. 69: 35[illegible]–371, 1967

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RELAPSING POLYCHONDRITIS

A Histopathological and Histochemical Study of the First Danish Case

By

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Received 20.viii.66

Only about 40 cases of relapsing polychondritis are on record. The present case is the first to be observed in Denmark. Its clinical aspects have been published previously (*Finn Jensen* 1962 a, b). Now a post-mortem examination is available, and as only 6 autopsied cases have been published (*Altherr von Meyenburg* 1936, *Harwood* 1958, *Kaliskin & Katzenstein* 1958, *Purcell, Nahum & Mcnell* 1962, *Verity, Larson & Madden* 1963 (2 cases)), it would seem of interest to report the pathological and histochemical findings which support the assumption that the disease entails a loss of acid mucopolysaccharides in the cartilage matrix (*Golden* 1963, *Verity, Larson & Madden* 1963).

The term relapsing polychondritis was introduced by *Pearson, Kline & Newcomer* (1960), but the disease has previously been described under a number of different names. In German literature it is usually called Polychondropathie or Chondromalacie.

The onset of the disease has occurred at ages ranging from 14 to 61 years. The primary clinical manifestation is recurrent inflammation of cartilaginous tissues, secondarily leading to multiple defects with collapse of the aural cartilages, the cartilages of the nose and respiratory tract, articular changes and sometimes inflammatory ocular lesions as well as involvement of the middle ear. The cause of death is usually respiratory insufficiency due to collapse of the air passages or possibly the sequelae thereof. The reported duration of the disease has varied from 1 to 24 years (*Kaye & Sones* 1964).

CASE REPORT

A h m [illegible] ...958 the patient was 45 years of age and th[illegible] pr[illegible] admission she had an in[illegible] ...atigue. [illegible] she ran a low grade [illegible] auricles which became bluish [illegible] the last month before admission she became hoarse, had pain on swall[illegible] [illegible] sweats, weight loss, chest pain and a dry cough. Moreover, she was comp[illegible] of pain in the metatarsophalangeal joints of both big toes and in the interp[illegible] joints of the ring and little fingers of the left hand.



Fig 1
The patient's right external ear at the time of the first admission

When admitted to the Medical Department of Skt Lukas Stiftelsens Hospital Copenhagen (case rec 718 58) she was on *physical examination* tired somewhat debilitated anaemic and emaciated Weight 43 8 kg height 158 cm

The external ear (Fig 1) except the lobule was found to be reddish cyanosed warm scaling with soft thickening of the subcutaneous tissue and partial loss of the scapha The *subcutaneous thickening continued into the external auditory* meatus which was almost obliterated The aural cartilages were tender and soft making the auricles droop The bridge of the nose was collapsed at the junction of bone and cartilage (Fig 2) The costal cartilages were tender and swollen at the junction of bone and cartilage reminiscent of a rachitic rosary There was moderate swelling of the metatarsophalangeal joints of both big toes and a more pronounced swelling of the interphalangeal joints of the left ring and little fingers The skin overlying the involved joints was rather reddened and the joints were tender

Laboratory findings E S R 119 mm/hour Hb 10 4 g/100 ml W B C 18 100 differential count showed relative lymphopenia The sternal bone marrow was the seat of uncharacteristic changes as seen in infections allergic diseases or Hodgkin's disease Serum iron 0 045 mg/100 ml serum uric acid 5 5 mg/100 ml W R and gonococcus reaction negative Mantoux (10 units + 8 mm sputum Tb negative on smears and culture No tumour cells in the sputum Serum electrophoresis revealed moderately reduced albumin values a slightly reduced α_1 slightly elevated α_2 and γ globulin values ECG Tachycardia but no other abnormalities in particular no signs of myocarditis Other laboratory tests showed no abnormalities X rays of toe joints Mild arthritic changes of the 1st metatars phalangeal joints on both sides Finger joints No changes of the interphalangeal joints Mild halisteresis

Biopsy from aural cartilage (Fig 3) The specimen was covered with normal skin The underlying thickened oedematous perichondrium showed severe infiltration by lymphocytes and plasma cells and a number of blood filled capillaries Towards



Fig 2

Patient's profile at the time of the first admission Note the saddle nose and the thickened somewhat flabby auricle

[illegible] was [illegible] was [illegible] [illegible] ...erative perichondritis with slight encroachment on the cartilage

Although the exact diagnosis was in doubt, there was obviously a question of a generalized disease, affecting mostly the cartilage It was not until later, when *Pearson et al* published their paper (1960), that it was realized that the patient's symptoms could be interpreted as a nosological entity

Further course The patient was put on prednisone which she continued to take until she died The maintenance dose was 5 mg twice daily On this dosage she was able to keep her full-time job as book keeper interrupted a few times a year by a week or two of febrile infections in the upper air passages Only once did she have to be admitted—with pneumonia in 1963

Terminal stage In the middle of December 1965 the patient again developed a catarrhal condition, with increasing respiratory embarrassment and difficulty in coughing up the tenacious sputum from the upper air passages The cough was insufficient owing to the softness of the cartilage A couple of hours after her admission (case rec 905/65) she developed respiratory arrest and shortly after that cardiac arrest She was immediately given external cardiac massage, and a spontaneous heart beat was re-established in about 3 minutes Thereupon she had tracheotomy and intubation and manual ventilation In addition an i.v. Aramine® drip was established and she had injections of Cedilanid® Solvisat® (cortisol) and penicillin A few hours later her condition had become so far stabilized that she could be transferred to the Bispebjerg H[illegible] [illegible] Department of Anaesthetics (case rec 411/65) for treatment in a resp[illegible] She [illegible] had to have artificial ventilation and parenteral nutrition The E[illegible] sh[illegible] signs of severe brain damage For some days the condition remained [illegible] nary but then she developed irreversible hypotension and anuria [illegible] days after admission without having regained consciousness at any t[illegible] the primary cardiac arrest



Fig 3

Biopsy from the auricle at the first admission The greatly thickened perichondrium with inflammatory reaction and small vessels occupies almost the entire picture The ill defined cartilage margin and the relatively intact cartilage may be seen superiorly Toluidine blue (× 500)

Autopsy (Bispebjerg Hospital, Dept of Path, aut rec R 34/66) 12 hours *post mortem*. *The body of a middle-aged woman whose nutri*tional state was below medium Typical saddle nose and deformity of the external ears which were soft to palpation Tracheostomy On the arms and legs large, plaque-shaped suggillations and on the left leg a scar after venous cut-down Joints normal to inspection The brain weighed 1520 g There was severe oedema of the cerebrum, and congestive haemorrhages were found in the brain stem Eyes pale and normal to inspection

Examination of the viscera showed the abdominal as well as retroperitoneal viscera to be normal apart from mild fatty degeneration of the liver cholecystitis and cholelithiasis The adrenals were normal

Examination of the thoracic organs showed a small patent foramen ovale but otherwise a normal heart Pleurae and lungs normal apart from pus in the bronchioles No pneumonic lesions and no atelectases

The main changes in the respiratory tract were softness and collapse of the epiglottis and laryngeal cartilages, moreover, the trachea had collapsed above the tracheostomy The mucous membrane was swollen, congested and necrotic in the right side of the trachea The trachea as well as bronchi were filled with mucopus When cut, the cartilaginous rings were soft to palpation

Histological and Histochemical Findings

MATERIAL AND METHODS

Considering that the main object of this investigation was histochemical examination of the cartilage to assess the condition of the acid mucopolysaccharides, tissue was removed from the auricles, epiglottis, trachea and bronchi. Both eyeballs were removed and fixed in Lillie's fixative for 50 hours, while the other specimens were fixed in 10 per cent buffered formalin for 24 hours, dehydrated, cleared in ligroin, embedded in paraffin and cut into sections 5 μ in thickness. In addition, normal eyeballs and normal cartilage from auricles, epiglottis, trachea and bronchi were used as control. The remaining biopsy material was regrettably too scanty for histochemical examination.

The following staining methods were employed:

(1) Haematoxylin eosin, (2) van Gieson Hansen, (3) Wilder's reticulum stain, (4) elastic tissue staining by Verhoeff's method (modified), (5) Alcian blue (AB) by the Hisson and Ekelund methods, (6) the PAS reaction after McManus (PAS), (7) Best's carmine, (8) PAS following diastase hydrolysis at 37° C for 60 minutes, (9) colloidal iron stain (CI) by the method of Barka, (10) colloidal iron + PAS (CI PAS), (11) 0.5 per cent aqueous toluidine blue (TBL) for 20 min, (12) 0.1 per cent azure A (AA) for 10 min at pH 2 to 5, (13) sulphation with sulphuric acid after Lewis & Grillo, followed by the Kramer & Windrum technique for metachromasia, (14) aldehyde fuchsin Alcian blue (AF AB) after Spicer & Meyer, (15) Alcian blue safranin O (AB S), (16) N N dimethyl *meta* phenylenediamine + N N dimethyl *para* phenylenediamine—Alcian blue (DI AB) by the method of Ley & Spicer (18 hours), and (17) mercury bromphenol blue staining (MBB).

RESULTS

Normal Cartilage

All control cartilages showed uniform reactions. However, staining of reticulin and elastic fibres was more pronounced in the elastic cartilage of the auricle and epiglottis than in hyaline cartilage. PAS positive reactions were found particularly in the cartilage matrix, but also more faintly in the cytoplasm of the chondrocytes. The PAS reaction was unchanged after diastase digestion, although Best's carmine showed quite pronounced staining of elastic as well as hyaline cartilage. Alcian blue gave a positive reaction only in chondrocytes, faint staining of the perichondrium, but deep staining of the lacunar rim. The same applied to colloidal iron which, however, stained the matrix faintly. Combined colloidal iron and PAS gave a blue colour to chondrocytes as well as to the lacunar rim and magenta to the matrix.

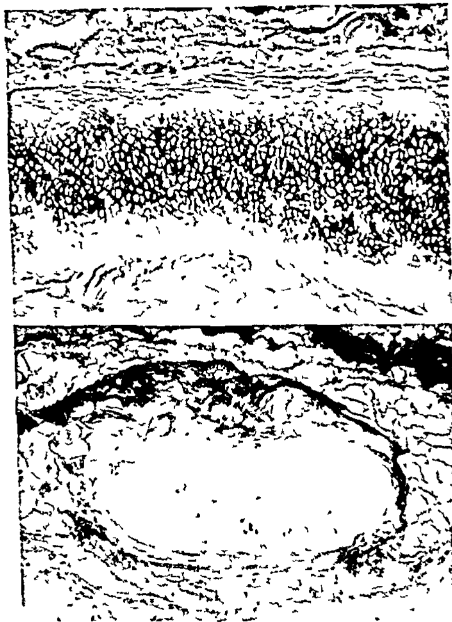
Aldehyde fuchsin Alcian blue, Alcian blue safranin and iron diamine Alcian blue showed deep staining of the matrix by aldehyde fuchsin and safranin O and black staining by iron diamine. Toluidine blue as well as azure A gave pronounced β metachromasia which with toluidine blue comprised the chondrogenic layer of the perichondrium but never reached the external layer. At pH 2 there was still a faint metachromatic reaction with azure A [illegible] rising to a maximum around pH 4. Following sulphation there was no definite increase in the staining intensity.

Mercury bromphenol blue [illegible] light staining of the matrix and the surrounding tissue also showed [illegible] the reaction.



Fig 4

Section from the abnormal cartilage of the trachea Note the pronounced defects in the cartilage, replaced by connective tissue In the defects there is still, especially in the lower picture, infiltration by inflammatory cells
Colloidal iron-PAS (top × 200, bottom × 500)



Fig

Flastic t ssue sta ning f normal (t p a i pat i l gical br nchial cartilage (I t tom) The latter h s c mplete tran f r nat n nto a connective tissue plate with out any fibrillar structur M l f ed Verhoeff (× 200)

TABLE 1

Staining Reaction of Normal and Pathological Cartilage

Staining method	Control	Auricle	Epiglottis	Trachea	Bronchi
Wilder	+++	+	+	—	—
Verhoeff (mod)	+++	+	+	—	—
PAS	+++	+++	+++	+++	+++
Best's carmine	++	++	+	+	+
PAS post diastase	+++	+++	+++	+++	+++
AB	+++*	+†	+†	—†	—†
CI	+++*	+†	+†	(+)†	(+)†
CI – PAS	+++*§	(+)	—	—	—
	+++	+++	+++	+++	+++
AF AB	+++§	+	+	(+)	(+)
	+	+	+	+	+
AB – S	+§	+	+	(+)	(+)
	+++	+	+	—	—
DI – AB	+++§	(+)	(+)	—	—
	+	+	+	(+)	(+)
TBI	+++	++	+	+†	+†
AA pH2	(+)	—	—	—	—
AA pH3	++	—	—	—	—
AA pH4	+++	+	+	+†	+†
AA pH5	+++	++	++	++†	+†
Metachromasia post sulphation	+++	+	+	+	+
MBPB	+	+	+	+	+

Symbols Staining method symbols See material and methods
— = no staining
(+)→+++ = degree of staining
* Reactivity only in chondrocytes, lacunar rims and perichondrium
† Reactivity present in the surrounding tissue and the regenerative connective tissue
§ Upper row Reactivity of the first dye in the sequence
Lower row Reactivity of the second dye in the sequence

Normal eyeballs showed a positive colloidal iron and Alcian blue staining in the layer of rods and cones as well as pronounced β metachromasia of the corneal stroma These methods caused less intense staining of the sclera, but a marked reaction in the vitreous body

Cartilage from the Autopsy Material

In general it may be said that the changes of the cartilage and cartilage remnants were of a chronic, fibrous nature unlike the exudative, inflammatory changes in the early stage of the disease seen in the biopsy specimen All the cartilage of the autopsy material was char-

acterized by severe changes. The margin was everywhere eroded. The resulting defects were replaced by collagenous connective tissue, in most sites without infiltration by inflammatory cells. In the trachea however there were remnants of inflammatory exudate in the major defects (Fig. 4). In several sites, especially in the bronchi, the cartilaginous tissue had entirely disappeared, being replaced by a connective-tissue plate whose outlines corresponded to those of the original cartilage (Fig. 5).

In the residual cartilage islands the number of chondrocytes had greatly decreased and there were several empty lacunae. Furthermore the fibrillar structure of the matrix demonstrated in normal cartilage by reticulum and elastic tissue stains had greatly decreased or been lost (Fig. 5).

Most interest attaches to the various methods for demonstrating mucopolysaccharides.

The metachromatic reactions were far weaker in the pathological cartilage but positive in the reparative connective tissue. However much depended on the pH, as no staining was obtained at the more acid reactions. In the normal cartilage β metachromasia was still present in a highly acid medium. There was no increase following sulphation. A characteristic finding of importance to pathogenetic considerations was the pronounced β metachromasia in the tissues surrounding the diseased cartilage, a reaction which was often as pronounced as in normal cartilage. This strong metachromatic reaction was in marked contrast to the weaker reaction in the reparative connective tissue and to the sharp demarcation of the metachromatic reaction in normal cartilage (Fig. 6).

As illustrated in Table 1 the other staining reactions were also greatly reduced in the pathological cartilage. However, colloidal iron as well as Alcian blue gave fairly pronounced diffuse staining of the reparative connective tissue and of the surrounding normal tissue. This is a considerable difference from normal cartilage in which these dyes stained mainly chondrocytes, lacunar rims and perichondrium. The reactivity was also far greater than in normal connective tissue. Aldehyde fuchsin Alcian blue and Alcian blue safranin also showed a blue staining of tracheal and bronchial cartilages, while there was a faint aldehyde fuchsin and safranin staining of the auricular and epiglottic cartilage. Iron diamine staining was negative in the pathological cartilage but there was a positive reaction at the site of bronchial glands. An extremely characteristic finding was the persistent PAS positivity in the residual cartilage matrix, while PAS positivity was not observed in the surrounding tissue or in the reparative connective tissue. PAS staining was unchanged after diastase hydrolysis.

Examination of the eyeballs revealed slight degeneration of the elastic fibres in the conjunctiva and in the left eye a small retinal detachment in the macular area. The histochemical reactions on the other hand showed no difference from the control eyes.

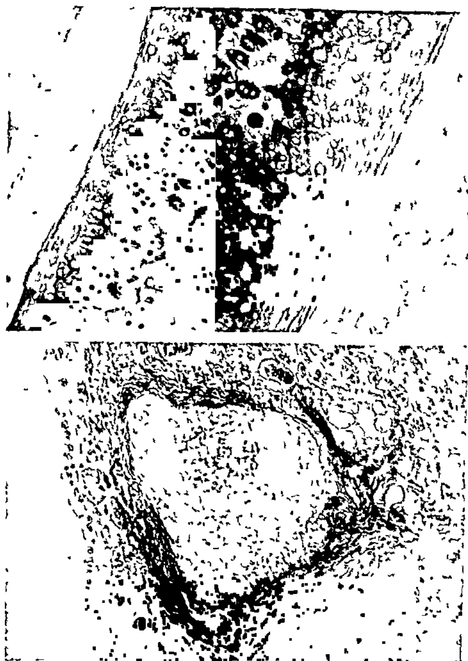


Fig 6

Metachromatic reaction in normal and pathological cartilage Note the sharp demarcation between the cartilage and surrounding tissue in the normal cartilage (top) and the faint staining in the central areas of the diseased tissue (bottom) On the other hand the bottom picture shows intense metachromatic staining of the surrounding tissue Azure A at pH 4 (× 200)

DISCUSSION

The clinical course and the pathological findings correspond accurately to previous cases published as relapsing polychondritis. *Verity, Larson & Madden* (1963) as well as *Kaye & Sones* (1964) have listed the affected organs. In practically all cases the cartilage of the auricles, nose and respiratory tract has been involved, but cartilage lesions may be observed in all parts of the body, including articular cartilage.

The eyeball and the heart, though non-cartilaginous, are strikingly enough often involved. This is possibly due to the high content of acid mucopolysaccharides of the eyeball and of the cardiac valves. The ocular changes manifest themselves as iridocyclitis or episcleritis. *Kaye & Sones* (1964) found the eyeballs to be involved in 24 out of 35 patients. The ocular manifestations have been described in detail by *Rucker & Ferguson* (1965).

Examination of the eyeballs from the present patient showed no pathological or histological changes which could be ascribed to polychondritis. And indeed, this was not to be expected, as there had been no clinical ocular symptoms.

As in most previous cases, the cause of death was increasing respiratory failure due to collapse of the respiratory tract. It is typical that nearly all the patients had been tracheostomized immediately before death.

Histological examination shows a characteristic development from exudative-inflammatory, mainly perichondrial changes with initially intact cartilage to a chronic fibrous stage. At this stage marginal erosion of the cartilage is seen, with breakdown of the matrix and chondrocytes, the defects being filled with connective tissue. At first this connective tissue is the site of moderate inflammatory reaction, later it is cicatricial. The histological changes observed in the biopsy specimen and at autopsy reflect this sequence of development.

The pathogenesis is elucidated by the histochemical examination of the cartilage.

The composition of the cartilage matrix has not yet been fully established (Handbuch der Histochemie 1964). It has been known for a long time that the matrix contains large quantities of chondroitin sulphuric acid. According to recent investigations (*Meyer et al* 1956) three quarters of the mucopolysaccharides are made up of chondroitin sulphate A, while chondroitin sulphate C is present in smaller quantities. There is not yet agreement as to whether chondroitin sulphate B is present. At any rate it is not mentioned as a component of cartilage by *Thompson & Hunt* (1966). Other components isolated from the ground substance are keratosulphate, a sulphated aminopolysaccharide (*Meyer et al* 1958) and chondroitin, a non-sulphated mucopolysaccharide (*Davidson & Meyer* 1954). *Chondroitin sulphate* A and keratosulphate are also present in large quantities in the cornea (*Meyer et al* 1953

No histochemical method is absolutely specific for the demonstration of acid mucopolysaccharides in general or for their individual subgroups in particular, but a combination of several methods may give an impression of the localization of these substances in the tissues. The reliability is increased if autoradiography with $S^{35}O_4$ is used for comparison. However, there is not agreement concerning the interpretation of the various reactions.

According to *Spicer* (1963) the purple and the red colour seen in the cartilage matrix with an aldehyde fuchsin-Alcian blue and an Alcian-blue-safranin sequence indicates a content of highly acid sulphated mucopolysaccharides. In the same way, *Lev & Spicer* (1965) state that sulphated mucosubstances stain black with iron diamine stains. The sulphated mucosubstances give pronounced β-metachromasia which, however, is given also by acid, non-sulphated mucopolysaccharides (*Wislocki et al.* 1947, *Spicer* 1963). All sulphated acid mucopolysaccharides do not, however, show metachromasia (*Spicer et al.* 1961). According to *Spicer* (1963) certain, non-sulphated acid mucopolysaccharides show orthochromasia.

A survey on the various sulphated acid mucopolysaccharides and their staining reactions has been given by *Spicer* (1960). According to *Spicer & Meyer* (1960) an aldehyde fuchsin-Alcian blue sequence is applicable for differentiating between sulphate and carboxyl groups.

Polysaccharides containing mainly carboxyl groups stain with colloidal iron and Alcian blue (*Mowry* 1963), while neutral mucopolysaccharides are PAS-positive and diastase resistant.

According to Table 1, highly acid sulphated mucopolysaccharides and neutral mucopolysaccharides appear to be present, especially in the ground substance, while chondrocytes, the perilacunar rim and the perichondrium contain particularly non sulphated acid and neutral mucopolysaccharides. A large part of the PAS-positive substances are presumably mucoproteins.

Table 1 shows, moreover, that the pathological changes increase downwards in the respiratory tract. The elastic cartilage in the auricle and epiglottis is involved to a lesser extent and the hyaline cartilage *of the bronchi most severely. In the most cranial part of the trachea the* hyaline cartilage is best preserved, though greatly eroded. These cartilage remnants still show PAS positivity, while reactions for acid mucopolysaccharides are negative. In the surrounding connective tissue, however, there is intense staining with Alcian blue and colloidal iron, but slight staining with aldehyde fuchsin. These findings presumably indicate leaking of acid mucopolysaccharides from cartilage and cartilage remnants to the surrounding tissues.

Two pathogenetic hypotheses have been suggested, *viz*:

(1) That the perichondrial inflammation is primary and the destruction of cartilage secondary.

(2) that the disease process affects the cartilage primarily, the perichondrial reaction being secondary.

These hypotheses have been thoroughly discussed by *Verity et al.* (1963). They conclude that most findings indicate that changes of the cartilage matrix with loss of acid mucopolysaccharides are primary. In this respect the finding of an increased urinary excretion of acid mucopolysaccharides is of interest (*Golden* 1963, *Rucker & Ferguson* 1965). This increased excretion is seen especially during relapses. In quiescent phases of the disease the urinary excretion of acid mucopolysaccharides has been found to be normal (*Kaye & Sones* 1964).

Experiments have shown that intravenous injection of the proteolytic enzyme papain into rabbits induces cartilage changes of a nature similar to that in human polychondritis (*Thomas* 1956, *Spicer & Bryant* 1957, *Westerborn* 1961). However, this has not led to ocular changes, only cartilage changes. Whether enzymatic defects play a role in the human disease still remains unelucidated.

Since similar cartilaginous changes, though of lesser severity, are observed in systemic lupus erythematosus, Hashimoto's thyroiditis, rheumatoid arthritis, and Sjogren's syndrome (*Kaye & Sones* 1964), and since corticosteroid therapy has a certain effect, some authors have classified the disease as a collagen disease, while others consider it to be an auto-immune disease.

The present study supports the hypothesis that acid mucopolysaccharides disappear from the cartilage matrix gradually as the disease progresses, but we are not prepared to say whether this is the primary defect or a secondary phenomenon. More advanced histochemical procedures (enzyme histochemistry, immune histochemistry, etc.) may possibly throw further light on the aetiology and pathogenesis of this disease.

SUMMARY

The first Danish case of relapsing polychondritis, previously described clinically, is now reported from the pathological and histochemical aspects.

The patient died in a way typical of the published cases, of respiratory failure following collapse of the upper air passages.

Histological examination revealed destruction of the hyaline cartilage increasing towards the distal end of the tracheo-bronchial tree. The elastic cartilage of the auricle and epiglottis appeared to be less severely involved.

Histochemical staining methods indicated a loss of acid mucopolysaccharides from the cartilage matrix. However, the present study did not afford a basis for concluding whether this is the primary defect or secondary to the early occurrence of perichondritis.

ACKNOWLEDGEMENTS

The authors acknowledge their indebtedness to *Willy Dam* M D, Head of the Department of Anaesthetics Bispebjerg Hospital for the loan of the case record from his department to Professor *Harald Gormsen* M D, for the investigation of the bone marrow, and to *Sven Petri* M D, Pathologist to the Copenhagen City Hospital for kindly leaving us the biopsy block For interesting technical and theoretical discussions thanks are due to Dr *Aage Johansen* and Dr *Hans Lyon* Department of Pathology, Bispebjerg Hospital

To *S Ry Andersen* M D, Head of the Ophthalmic Pathology Laboratory we are grateful for help in examining the eyes

Moreover we are thankful for the technical assistance of Mrs *Grethe Kongsmar* and Miss *Jette Pedersen* Department of Pathology Bispebjerg Hospital and of Mrs *Tove Jacobsen* the Ophthalmic Pathology Laboratory

The study was aided by a grant from *Cold Stores Fund* Copenhagen for which we extend our thanks

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DIFFERENTIATION OF LARGE VACUOLATED NEOPLASTIC CELLS IN EPIDERMIS BY MEANS OF PAS STAINING

By

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Received 25.xi.66

Distinction between Paget cells and neoplastic cells from melanomas penetrating the epidermis as described by *Allen & Spitz* (1953) may sometimes cause difficulties to the pathologist, as pointed out by *Lever* (1961). The difficulties in distinguishing Paget's disease from early melanoma have been demonstrated by *Culberson & Horn* (1956), who found that Paget cells may contain melanin. It was reported by *Fisher & Beyer* (1959) that periodic acid Schiff positive material could be identified in the cytoplasm of many of the large intraepidermal cells of Paget's disease in all lesions investigated, while this was not the case of melanomas. In the latter lesion cells containing melanin when stained by this technique frequently showed a content of rust coloured cytoplasmatic material. In contrast to the slight PAS positive material which may be found in the surrounding squamous cells, the PAS positive material in Paget cells does not disappear after diastase digestion. From these studies it seems as if the PAS staining may provide means of distinguishing between abovementioned lesions. On the other hand it appears that such an assumption would build on 8 cases of melanomas and 3 cases of Paget's disease. The author has therefore attempted to test the differentiation by means of PAS staining on a larger material.

MATERIAL AND METHODS

The present material consists of 36 cases of Paget's disease and 25 malignant melanomas. Sections were prepared from formalin fixed blocks and stained by the periodic acid Schiff reaction, haematoxylin-eosin and alcian blue staining.

RESULTS

The results of the tinctorial reactions are listed in Table 1. It is seen that no intraepidermal cells of malignant melanomas contained PAS positive material in contrast to Paget's disease among which 9 cases

out of 36 showed intraepidermal tumour cells containing small granules of distinct PAS positive material. One case appeared to demonstrate several large neoplastic cells in the epithelium showing a considerable amount of PAS positive material. It is further noted that none of the sections from Paget's disease as well as from melanomas contained distinct Alcian blue positive material in the cytoplasm of the large intraepidermal tumour cells.

TABLE I

Staining Reactions of the Cytoplasm of Large Intraepidermal Neoplastic Cells in Paget's Disease and Malignant Melanomas

No. of cases		PAS Reaction Positive/Negative		Alcian Blue Reaction Positive/Negative	
36	Paget's disease	9	27	0	36
25	Melanomas	0	25	0	25

DISCUSSION

The utilization of histochemical procedures may proove valuable in tumour differentiation as shown by *Burstone* (1961). The differentiation by means of PAS-positive material in the large vacuolized intraepidermal cells of Paget's disease have, however, in this material been of less value as 75 per cent of the cases studied appeared to have no PAS positive material in the large, optically clear cells situated in the epithelium. This is in contrast to the material presented by *Fisher & Beyer* (1959) who found PAS positive material in all lesions investigated. None of the melanomas appeared to contain PAS positive substance but often a rusty coloured material were found in cells containing melanin which correspond with *Fisher & Beyer* (1959) and *Pearse* (1961). None of the 61 neoplastic lesions showed a positive alcianblue staining.

SUMMARY

Thirtysix cases of Paget's disease and 25 malignant melanomas were investigated by means of PAS and alcian blue staining techniques as to their contents of PAS positive material in the large vacuolated intraepidermal cells found in the neoplasms mentioned. Only 9 cases of Paget's disease out of 36 were found to present PAS positive material in the large intraepidermal cells, while none of the melanomas gave positive reaction. The alcian blue staining was negative in all cases investigated. The PAS staining may thus be considered a useful means in differentiating between certain cases of Paget's disease and malignant melanomas. A negative reaction, however, does not exclude the presence of Paget's disease.

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HUMORAL IMMUNITY DURING THE INDUCTION OF EXPERIMENTAL AMYLOIDOSIS

By

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Received 22.viii.66

That immunological phenomena very probably play an important role in the development of experimental amyloidosis is generally accepted. It is well known that a wide range of materials are effective in inducing amyloidosis in many species, especially if such materials are capable of acting as antigens themselves or indirectly if they induce suppuration in the treated organism. In histological investigations on amyloidosis in mice subjected to hyperimmunization with casein it appeared that the formation of amyloid in the tissues depended on a biphasic mechanism: an initial pre-amyloid phase with proliferation of reticuloendothelial pyroninophilic cells and plasma cells and rise in the serum gamma globulin, a second amyloid phase with decreasing pyroninophilia and the appearance in rapid succession of P.A.S.-positive reticuloendothelial cells in the border zone of new formed amyloid deposits (*Teilum* 1956, 1964 a). This cytochemically well defined transition from a preamyloid to an amyloid phase was believed to be the result of an exhaustion leading to a functional break-down of the immune process responsible for the initial pyroninophilic phase, the exhaustion being the consequence of a protracted and heavy antigenic stimulation. These studies showed a definite functional relationship between reticular cells and amyloid, but the type of immune reaction apparently taking part in the pathogenesis of amyloidosis remains ill defined. Based on the assumption that this alleged immune dysfunction triggering the amyloid phase might reflect itself in a general disturbance in one or more of the immune mechanisms, we decided to investigate the effect of an amyloid inducing regimen on the major host defence mechanisms: *phagocytic activity, delayed (or cellular) hypersensitivity*, and the capacity to form circulating antibodies. In functional studies of the phagocytic properties of the R.E.S. during the development of casein induced amyloidosis in mice phagocytosis was found to vary with the

These investigations were supported by grants from *The Danish League Against Rheumatism* and *The General Scientific [illegible] of The Danish Government*.

I am grateful to [illegible] Dr. *Joan Madsen*, [illegible] Statens Seruminstitut, Copenhagen, for help and advice regarding the choice of antigen and the antibody titrations.

phasic development of the disease the pyroninophilic, preamyloid phase was accompanied by a steady increase in phagocytosis of colloidal carbon, while the second, amyloid phase coincided with a decrease in phagocytic activity (*Ranløv* 1966b). Another experiment showed a significant delay in skin homograft rejection in casein-treated mice during the amyloid phase (*Ranløv & Jensen* 1966).

The present experiment is an attempt, under similar conditions, to determine the capacity to form circulating antibodies towards another antigen, ferritin, in groups of mice primarily treated with casein for different lengths of time.

MATERIAL AND METHODS

72 closely inbred C3H mice of equal sex distribution were used. They were from 10 to 15 weeks old at the beginning of the experiment. They were fed on ordinary food pellets and water except during the casein treatment when they were fed on oats. After randomization they were divided as follows:

12 control mice
12 mice treated with casein for 1 week (6 injections)
12 mice treated with casein for 2 weeks (12 injections)
12 mice treated with casein for 4 weeks (24 injections)
12 mice treated with casein for 6 weeks (36 injections) and
12 mice treated with casein for 8 weeks (48 injections)

Pretreatment. 0.5 ml of a 5 per cent solution of casein in 0.25 per cent sodium hydroxide was injected subcutaneously in different sites of the back daily, 6 times a week.

Ferritin immunization. The day following the last casein injection (in the untreated controls on the first day of the experiment) each animal received an intraperitoneal injection of 0.1 mg horse spleen ferritin (Fluka A.G.) in 0.5 ml sterile saline mixed with an equal volume of Freund's complete adjuvant (Difco). After 7 days each animal was given an additional dose of 0.1 mg ferritin in 0.5 ml sterile saline intraperitoneally. 6 days after the booster injection the animals were killed by heart puncture under ether anaesthesia. The blood was allowed to clot for half an hour at 37° C, centrifuged, and serum was stored at −20° C.

Pathology. Lung, thymus, liver, spleen, kidney, adrenal, ilium and mesenteri[illegible] lymph node were fixed overnight in 10 per cent neutral formalin, paraffin embedd[illegible] and sections were cut 5 microns thick. They were stained with methyl green pyronin and with the P.A.S. technique.

Antibody titration. The titre of antiferritin antibody was estimated in seri[illegible] dilutions of each mouse serum beginning at 1:20. A passive haemagglutination technique with formalinized sheep red blood cells was used, all sera being analysed in the same procedure on the same day.

Sheep red blood cells were washed 6 times in cool saline and made up to an 8 per cent suspension to which was added an equal volume of a 3 per cent solution of formaldehyde in saline. This mixture was left overnight at 37° C, washed 6 times and resuspended in saline. A 10 per cent suspension was stored at 4° C. 2 ml of the suspension was washed once in 40 ml phosphate buffered saline (PBS) (phosphate buffer 0.05 M, pH 7.0 and normal saline 1:4) and resuspended in 40 ml PBS. A mixture of 4 ml freshly prepared tannic acid (0.1 per cent) and 56 ml PBS was added, after which the mixture was left at 37° C for 15 minutes, centrifuged in the cold for 5 minutes at 4000 r.p.m. The wash was repeated with 100 ml of PBS, the mixture was resuspended in 50 ml of PBS followed by the addition of a freshly prepared solution of 10 mg of ferritin in 50 ml PBS. The sensitization was carried out at 37° C for 30 minutes. After centrifugation, resuspension in 100 ml PBS and centrifugation, the sensitized red cells were washed in 100 ml PBS containing 3 per cent normal rabbit serum, the latter previously being absorbed twice with formalinized sheep red blood cells. Finally the sensitized sheep red blood cells were

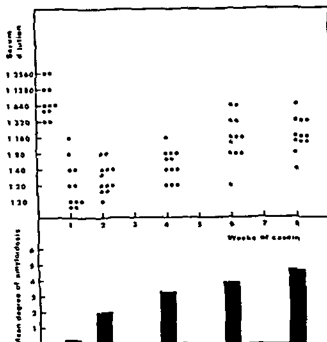


Fig. 1

Serum anti ferritin antibody titres (top) and mean degrees of amyloidosis (bottom) in groups of mice pretreated with casein for different lengths of time. The titres at the time "0" represent non casein treated controls.

made up and stored as a 10 per cent suspension in PBS with 1.5 per cent absorbed normal rabbit serum.

0.2 ml of the serum dilution was mixed with 0.1 ml of a 2 per cent suspension of sensitized sheep red blood cells and left overnight at 4° C. The reactions were read as –, +, ++, +++ and ++++ respectively. The titre was expressed as the dilution in the last well showing positive haemagglutination (+ considered negative).

As negative controls were used normal rabbit serum and PBS. As positive control a rabbit serum of known titre.

RESULTS

The main results of the experiment are graphically outlined in Fig. 1. The spleen being the primary and most constant site of amyloid formation in our system (*Christensen* 1963), sections of this organ were chosen for the purpose of estimating semiquantitatively the degree of amyloidosis as described by *Christensen & Hjort* (1959). However it should be emphasized that the mean degrees of amyloidosis for each of the groups presented in Fig. 1 do not truly represent the amounts of amyloid present in the spleens after termination of the casein treatment but represent the amounts of amyloid present in the spleens after additional 2 weeks of ferritin immunization. According to experience amyloid formation stops with the termination of casein administrat

and reabsorption does not take place (*Christensen* 1963) On the other hand, injections of Freund's adjuvant alone or in combination with other antigens may cause amyloidosis (*Christensen* 1962) In the present material the combined treatment with ferritin and Freund's adjuvant probably caused the minute amounts of amyloid observed in one of the 12 control mice Thus small amounts of amyloid have probably been added to every group of animals as a side effect of the ferritin immunization However, as it does not influence the conclusions of the present experiment no attempts of correction have been made With this modification the variations in mean degrees of amyloidosis between the various groups corresponded well to those observed under similar conditions in earlier experiments (*Ranløv* 1966b, *Ranløv & Jensen* 1966) Significant amounts of splenic amyloid were not seen until 2–3 weeks after the initiation of the casein treatment After this the spleen amyloidosis gradually increased, concurrent with increasing amyloid infiltration of the periportal areas of the liver, the renal glomeruli, the lungs and, finally, the inner layer of the adrenal cortex

In Fig 1 the anti-ferritin antibody titre for each single animal is plotted against the lengh of the casein treatment Among the controls the lowest titre was 320, the majority of controls being between 640 and 1280 In the group receiving casein for one week prior to ferritin immunization the mean antibody titre had fallen to a value between 20 and 40 4 animals showing titres below 20 After 2 weeks of casein a slight increase occurred, the mean titre still being between 20 and 40 but only one animal showed a titre lower than 20 After 4, 6 and 8 weeks of casein a steady increase towards control values of anti-ferritin antibody titres was evident, the titre at 8 weeks being between 160 and 320 No relation between antibody titre and degree of amyloidosis in individual animals was noted

DISCUSSION

A possible participation of immunoglobulins and circulating antibodies in the pathogenesis of amyloidosis has been the subject of much speculation *Loeschcke* (1927) regarded amyloid as a precipitate of antigen-antibody complexes and modified versions of this view have later been put forward (*Letterer* 1934, *Latvalathi* 1953) However, investigations by *Vazquez et al* (1957) and *Calkins et al* (1960) have not been able to confirm such hypotheses Further, no correlation between the amount of serum anti-casein antibody and degree of amyloidosis has been shown to exist in rabbits (*Giles & Calkins* 1958) or in mice (*Ranløv & Ebbesen* 1965) Most investigations of the serum proteins during the induction of experimental amyloidosis have shown a steady increase in the gamma fraction regardless of the amount of amyloid formed as a result of the amyloidogenic treatment (*Giles & Calkins* 1958, *Clausen* 1960, *Christensen & Rask-Nielsen* 1962, *Ranløv* 1966a) In the present

experiment the capacity to form circulating antibodies returned towards normal with increasingly severe amyloidosis. Compared with the observations of amyloidosis developing in agammaglobulinaemic individuals showing the antibody deficiency syndrome (*Gray et al.* 1951, *Teilum* 1964b, *Squire* 1965) the above mentioned and the present observations seem to argue against any definite relationship between humoral immune mechanisms and mechanisms involved in amyloid formation.

Different cells or cellular mechanisms seem to be concerned in the synthesis of circulating antibody and in cellular hypersensitivity reactions. Hypo- and agammaglobulinaemic individuals usually respond well with delayed hypersensitivity reactions in spite of an almost total lack of plasma cells and ability to produce circulating antibodies (*Porter* 1955, *Kelly et al.* 1958, *Arsenberg & Leskowitz* 1963). On the other hand, patients with reticuloses such as sarcoidosis or Hodgkin's disease usually produce circulating antibodies normally while frequently failing to develop delayed hypersensitivity reactions (*Rostenberg* 1951, *Sokal & Primikirios* 1961).

In contrast to the findings in the present experiment of an increasing ability to form circulating antibodies with increasing amyloidosis is the decrease in phagocytic capacity with increasing amyloidosis found in rabbits (*Shearing et al.* 1965) and in mice (*Hanlon* 1966b). This may indicate a functional relationship between amyloid producing mechanisms and reticuloendothelial cells corresponding to the earlier described relationship (*Teilum* 1956). In the same way the fact that homograft rejection is inhibited with increasing amyloidosis (*Hanlon & Jensen* 1966) seems suggestive of a participation of immune reactions akin to the cellular delayed type in the pathogenesis of amyloidosis. As it is well known that delayed hypersensitivity reactivity such as allergic encephalomyelitis (*Astrom & Waksman* 1962), experimental allergic thyroiditis (*Felix Davies & Waksman* 1961) and experimental allergic nephritis (*Hess et al.* 1962) may be transferred to normal recipients with spleen or lymph node cells, it is of interest to note that experimental amyloidosis in the same way has been transferred to normal syngeneic recipients by means of injections of spleen cells from casein treated donor mice (*Werdelin & Ranlov* 1966).

In contrast to the almost normal antibody response after 6 and 8 weeks of casein treatment is the marked suppression of the same response observed in mice which have received casein for only one or a few weeks prior to the ferritin stimulation (Fig. 1). The reason for this temporary suppression is not obvious, but it seems most likely to be the result of an antigenic competition between casein and ferritin.

Ant[illegible]ic c[illegible]mpetition is generally accept[illegible] [illegible]s a real imm[illegible]n[illegible]l[illegible]gic phen[illegible]me[illegible]n wh[illegible]c[illegible] under appropr[illegible]ate conditions ca[illegible] [illegible]e [illegible]demonstrated with many different types [illegible]f ant[illegible]gens. Among ot[illegible]ers *M[illegible]ller et [illegible]l.* (1[illegible]61) descri[illegible]ed severe mut[illegible]al re[illegible]c[illegible]tions in the antib[illegible]dy r[illegible]sponses to [illegible] different [illegible]acterial an[illegible] protein anti[illegible]ns w[illegible]en

injected simultaneously into the same mouse They further described the delay in skin homograft rejection after large doses of other antigens presumably unrelated to the transplantatio[illegible] in guinea pigs may [illegible] bacteria (*Rapaport* [illegible] rently injected with [illegible] tion mainly acting in reducing the primary response

In chickens it has been shown that the simultaneous injection of bovine serum albumin (BSA) and human gamma globulin (HGG) did not result in significant reductions in either antibody response However, if the injection of the same amount of HGG was delayed for 24 hours after the BSA injection the antibody response to the latter antigen was significantly reduced (*Abramoff et al* 1961). In guinea pigs a primary stimulus of diphteria toxoid suppressed the response to a course of two injections of tetanus toxoid when the first of these was given at times ranging from 3 to 21 days later, the "competetive" effect being maximum at day 7 (*Barr & Llewellyn-Jones* 1955) Thus it seems that the timing of the different antigenic stimuli is critical and specific for the various experimental systems employed

Very probably the dosage, too, is critical *Adler* (1964) showed that small doses of bovine gamma globulin given together with ferritin enhanced the antibody titre to ferritin while larger doses of bovine gamma globulin depressed it In this connection it should be emphasized that the single and the accumulated dosage of *casein administered to the various groups in the present experiment* was considerably larger than were the amounts of "competing" antigen employed in other experiments reported in the literature, in the present material approximately 1 000 mg per kg body weight (daily) as compared to 20–50 mg in single or a few doses (*Abramoff et al* 1961 *Rowinski et al* 1966) That the inhibition of the response to a second unrelated antigen may be proportional to the amount of "competing" antigen administered has been regarded as supporting a concept of antigenic competition being a *competition for antibody-producing cells or their precursors* (*Adler* 1964) Such a "clonal" concept may easily explain the markedly depressed anti ferritin antibody response in the present material after one or two weeks pretreatment with casein as a "crowding out" effect—most of the available antibody producing cells being engaged in coping with the casein However it offers no explanation of the rising anti-ferritin antibody titre following larger amounts of casein The reason for this discrepancy is not apparent, it might be attributed to a slowly developing state of tolerance towards casein, or it may be caused by a slowly developing compensatory increase in the number of non committed clones

In summarizing the results of the present and earlier reported experiments on the functions of the immune mechanisms during long-standing antigenic stimulation leading to the development of amyloidosis in mice it may be stated that 1) the primary, pyroninophilic preamyloid phase is characterized by a) a rapid increase in phagocytic capacity (*Ranlov* 1966b), a probably normal cellular hypersensitivity reactivity (*Ranlov & Jensen* 1966) and c) a rapid decrease in the capacity to form humoral antibody against other antigens 2) The second amyloid phase is characterized by a) a steady and marked decrease in phagocytic capacity (*Ranlov* 1966b), b) a significantly reduced cellular hypersensitivity reactivity (*Ranlov & Jensen* 1966) and c) a normalization of the capacity to form humoral antibodies against other antigens in spite of an increasingly severe amyloidosis These findings seem to imply a participation of reticuloendothelial cells and immune mechanisms akin to the delayed or cellular type in the pathogenesis of experimental amyloidosis

A number of other investigations support the assumption of a connection between reticular tissues, cellular immune reactivity and amy-

loidosis. Graft rejection and graft versus host reactions are both believed to rely on cellular immune mechanisms and both are mediated through large pyroninophilic cells (*Gowans* 1962) indistinguishable from the large pyroninophilic reticular cells constantly seen dominating the tissues during the preamyloid phase. Small lymphocytes, believed to be active in cellular immune reactions, are deficient in the peripheral blood during the amyloid phase (*Latvalahti* 1953, *Christensen & Rask-Nielsen* 1962). In this connection it is noteworthy that the large pyroninophilic cells of Gowans have been shown to be the result of transformation of small lymphocytes. The fact that immune reactivity of the cellular (or delayed) type may be transferred with spleen or lymph node cells while humoral immune reactivity can not, correlated to the observation that amyloidosis can be produced by transplantation of spleen cells from casein treated mice to normal syngeneic recipients (*Werdelin & Ranløv* 1966) lends further support to the assumption of cellular immune reactivity playing a role in the pathogenesis of amyloidosis.

SUMMARY

In continuation of earlier investigations of the influence of an amyloid inducing regimen on phagocytosis and delayed hypersensitivity reactivity in mice an experiment under similar conditions has been carried out in casein treated mice in order to define their capacity to form humoral antibodies against another antigen, ferritin, at various stages of the amyloid induction.

It was found that mice treated for one week with casein showed a marked decrease in serum anti-ferritin antibody, the titre being lowest at this stage. After 2 weeks of casein pretreatment the titre began to rise. With increasingly severe amyloidosis, after 4, 6 and 8 weeks of casein, a steady increase in the capacity to form anti-ferritin antibody was evident, the response after 8 weeks approaching control values. This temporary suppression of humoral antibody response is discussed as a phenomenon of antigenic competition.

Based on earlier observations of decreasing phagocytic capacity and delayed homograft rejection with increasing amyloidosis in mice, in contrast to the normalizing humoral antibody response found in the present material, a participation of reticuloendothelial cells and immune mechanisms akin to the delayed or cellular type in the pathogenesis of amyloidosis is suggested.

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REACTION OF ANTINUCLEAR FACTORS WITH POLYMORPHONUCLEAR GRANULOCYTES

2 *On the Inaccessibility of Intact Leucocytes to Granulocyte-Specific ANF of High Molecular Weight*

By

PREBEN ELLING

Received 26 viii 66

Antinuclear factors (ANF) which reacted selectively with granulocytes in human blood smears, but not with other mesenchymal or epithelial cell nuclei, have been found in some sera as the only ANF detectable by the immunofluorescent antibody technique (8, 16) Granulocyte-specific ANF have been demonstrated mainly in sera from patients with arthritic lesions, especially rheumatoid arthritis (10), though absorption studies also have revealed the existence of granulocyte-specific ANF in sera from patients with systemic lupus erythematosus, containing apparently organ-non-specific ANF reacting with all kinds of nuclei (6)

Wide variations in the incidence of ANF reacting with leucocytes in human blood smears have, however, been reported (*Feltkamp* (11) 12 per cent, *Barnett et al* (2) 30 per cent; *Hymans et al* (13) 41 per cent, *Alexander et al* (1) 65 per cent *Hasker et al* (12) 70 per cent *Elling* (7) 75 per cent), but since special precautions have to be taken to ascertain the maximum antigenicity of granulocytes, this may probably explain the marked differences found The main point seems to be that alteration of the granulocytes, *e g* by freezing the smears prior to testing for ANF, caused a considerable increase of the incidence of granulocyte-reactive ANF in sera from patients with rheumatoid arthritis Thus in a previous report (7) it was found that while 75 per cent of the sera from patients with rheumatoid arthritis reacted with altered granulocytes, only 30 per cent of the same sera reacted with unaltered leucocytes Similarly, only 35 per cent of the sera reacted with other human nuclei indicating that ANF in rheumatoid arthritis exhibit a limited nuclear reactivity, mainly directed against the polymorphonuclear granulocytes

Since the increased reactivity of altered granulocytes might indicate an inaccessibility of the living intact leucocytes to some ANF, the present study was conducted with the object of investigating the inter

action of ANF with polymorphonuclear granulocytes of various ages and degrees of alteration employing sera containing apparently only granulocyte-specific ANF as the analytical "tool". Since inaccessibility of the intact leucocytes may be related to the molecular size of the antibody (11), special emphasis has been laid on the reaction of leucocytes with ANF of different molecular weights.

MATERIAL AND METHODS

The material consisted of 20 sera shown to contain ANF reacting with polymorphonuclear granulocytes only, as determined by the immunofluorescent antibody technique. To ascertain the organ specificity, the 20 sera were tested with the following human tissues: thyroid gland, normal liver, gastric mucosa, striated muscle and renal cortex.

Fourteen sera were derived from patients with rheumatoid arthritis, one serum from a patient with Felty's syndrome, 2 sera from patients with systemic lupus erythematosus and 3 sera from patients with other non rheumatic diseases.

Methods. The fluorescent antibody technique for the determination of ANF was performed as previously described (6, 9, 10) with the following modifications.

Blood smears used as nuclear source were prepared from the same human donor in 3 different ways. 1) Blood smears were prepared immediately after drawing the blood and the sera were applied to the smears within 15 minutes. 2) Blood smears were prepared as in 1) but immediately frozen by placing them on dry ice for a few minutes and then thawed at room temperature, repeated once or twice. 3) after drawing the blood was shaken vigorously for 10 minutes in tubes containing glass beads. Smears were then prepared as in 1).

Rabbit antisera against human gammaglobulins were prepared and conjugated with fluorescein isothiocyanate as described (9). Specific antisera against human Ig G, Ig A and Ig M immunoglobulins were obtained from the Central Laboratory, Rode Kruis, Amsterdam, and conjugated with fluorescein isothiocyanate. The immunochemical specificity of these antisera was tested as described previously (f

The sulphydryl sensitivity of ANF was determined as described (10) with the use of 1 methyl-cystein. Control were diluted in buffered saline.

EXPERIMENTS AND RESULTS

Nuclear Reactivity of Blood Smears of Various Ages

With the use of blood smears less than 15 minutes old, 18 of the 20 sera employed reacted each time tested (Table 1), while 2 sera were positive in 1 out of 3 tests only. Repeating the reactions 5-6 times with each of the 18 positive sera they were found to react in every test showing that the use of fresh blood smears yielded fairly reproducible results.

With the use of blood smears 30 minutes old, altogether 6 sera became negative, including the 2 sera showing varying positive and negative reactions with 15 minute-old ... rs. Employing blood smears 60 minutes old, the 6 sera were still ... gative but 2 other sera showed inconstant positive and negative r... t

The finding that the same 6 s... r... constantly negative when 30 and 60 minute-old smears were u... nuclear source indicates that the non reactivity is not entirely ... dent on changes of the ... cytes owing to age but might ... the serum factors being ...

higher molecular weight, for example The finding that the antigenicity of these old smears was restored for 4 of the 6 negative sera by freezing the 60-minute-old smears prior to testing for ANF, supports this view

TABLE 1
Nuclear Reactivity of Human Blood Smears of Various Ages with 20 Sera Containing Granulocyte Specific ANF

	Human blood smears								
	15 minutes old			30 minutes old			60 minutes old		
	pos	neg	dubious pos.	pos	neg.	dubious pos	pos	neg	dubious pos
Number of sera	18/20	0/20	2/20	14/20	6/20	0/20	12/20	6/20	2/20
Number of tests performed with each serum	5 6	–	3	1	1	1	2	2	2

TABLE 2
Effect of Alteration of Blood Smears Prior to Testing for Granulocyte-Specific ANF in 20 Sera Compared with the Results Obtained with Fresh Unaltered Blood Smears

Human blood smears					
Unaltered 15 minutes old		Altered by freezing × 3		Altered mechanically	
pos	neg	pos	neg	pos	neg
20/20	2/20*	19/20	1/20	8/8	0/8

* 2 sera inconstantly positive

Nuclear Reactivity of Leucocytes Altered Mechanically or by Repeated Freezing and Thawing

The reaction of sera with leucocytes altered by freezing, compared with the reaction of sera with 15-minute-old, unaltered leucocytes, is seen in Table 2 Of the 20 sera containing leucocyte-specific ANF 17 reacted equally well with blood smears frozen and thawed 3 times as with fresh, intact leucocytes The 2 sera reacting inconstantly with unaltered leucocytes showed positive reactions with altered leucocytes while one serum did not react with altered leucocytes To obtain maximum antigenicity or accessibility, freezing and thawing the blood smears only one or two times proved insufficient, since only 12, respectively 14 out of 19 sera tested gave positive reactions All of 8 sera tested against leucocytes altered by shaking the blood with glass beads reacted, thus showing that a simple mechanical alteration of the leucocytes produced the same optimal antigenicity as repeated freezing did

Determination of the Immunoglobulin Classes of Granulocyte-Specific ANF, Employing Leucocytes Altered in Various Ways

To test whether the non-reactivity of old leucocytes to some ANF found in the present study was in any way related to the molecular size of the antibody, the immunoglobulin classes of granulocyte-specific ANF were determined by the use of blood smears altered in various ways and employing specific, conjugated antisera against human Ig G, Ig A and Ig M immunoglobulins.

TABLE 3

Immunoglobulin Classes of Granulocyte-Specific ANF in 20 Sera Tested Against Altered and Unaltered Leucocytes as Nuclear Antigen

Antigen	Immunoglobulin class Ig G	Ig A	Ig M
15 min old unaltered blood smears	17/20 (11)	4/20 (1)	5/20 (2)
Blood smears altered by freezing × 3	17/20* (2)	7/20* (0)	15/20 (2)

In brackets: Number of sera containing ANF of one immunoglobulin class only.
* 1 serum constantly negative.

Table 3 shows that with the use of 15 minute-old, unaltered leucocytes, 17 of the 20 sera contained Ig G ANF, 4 contained Ig A ANF and 5 sera contained Ig M ANF. 14 of the sera thus contained ANF belonging to one immunoglobulin class and 6 sera contained ANF of more than one immunoglobulin class.

Employing blood smears altered by freezing 3 times, 3 more sera were found to contain Ig A ANF and 10 more sera to contain Ig M ANF, while the frequency of low molecular weight Ig G ANF was exactly the same as found with the use of 15 minute-old, unaltered leucocytes. Only 4 sera now contained ANF of one immunoglobulin class.

The results thus show that the use of altered leucocytes increased the detection of high molecular weight ANF, while ANF of low molecular weight (Ig G) reacted with intact as well as with altered granulocytes to the same extent.

The question arises that the high molecular weight ANF found to react with altered granulocytes only might not be organ specific and might react with other nuclei altered in the same way. This was partially excluded since none of 8 sera tested reacted with thyroid nuclei altered by repeated freezing and thawing, and no reactions were observed with lymphocytes treated in the same way as the granulocytes.

TAB

Sulfhydryl Sensitivity of Leucocyte Specific ANF in 6 Sera and of Organ No

Patients	C			N			N J		
Immunoglobulin classes	Ig G	A	M	Ig G	A	M	G	A	Ig M
Nuclear activity before	+++	0	0	+++	0	0	0	0	+++
and after treatment with dimethylcystein	+++	0	0	+++	0	0	0	0	0

Sulfhydryl Sensitivity of Granulocyte-Specific ANF

To test the correlation between the sulfhydryl sensitivity of granulocyte-specific ANF and the immunoglobulin classes of ANF as determined by the use of specific antisera against human Ig-G, Ig A and Ig-M immunoglobulin classes, 6 sera were selected from the 20 containing granulocyte-specific ANF. Two sera contained apparently only Ig-G ANF, 2 only Ig-M ANF, and 2 contained ANF of both Ig G and Ig-M specificity. Furthermore, as a control, one serum from a patient with S.L.E., containing organ-non-specific ANF belonging to the three immunoglobulin classes and with a titre of 1:400 (tested with use of a conjugated antiserum against all human gamma-globulins and with use of thyroid nuclei), was tested for ANF before and after treatment of sera with dimethyl-cystein.

Table 4 shows a fair accordance between the sulfhydryl sensitivity and the immunoglobulin classes as determined by the use of specific conjugated antisera. The Ig-G ANF was unaffected except for a small

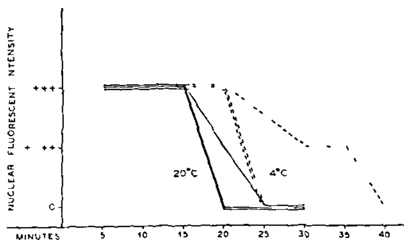


Fig. 1

Nuclear reactivity of blood smears of various ages with 3 sera containing granulocyte specific ANF. The reactions were performed at 20° C and 4° C.

...cific ANF in ... Serum (B) as Determined with Conjugated Specific Antisera

M			S G.			J			B		
Ig			Ig			Ig			Ig		
G	A	M	G	A	M	G	A	M	G	A	M
0	0	+++	+++	0	++	+++	0	+++	+++	+++	+++
0	0	0	++	0	0	++	0	0	+++	+	+

decline in the intensity of the fluorescence in 2 sera The Ig M ANF activity was abolished entirely except in the case of the lupus serum in which however a considerable fall in the intensity of the fluorescence was observed The Ig A ANF showed a similarly great decrease in fluorescent intensity after incubation with dimethyl cystein

Effect of Varying the Reaction Temperature

Eight sera containing granulocyte specific ANF were incubated with unaltered blood smears 15 minutes-old at 4°, 20° and 37° C, for half an hour No difference in nuclear staining was found but Fig. 1 shows that performing the reaction at a low temperature increased the limiting time factor from 15 minutes to 35 minutes in one case

DISCUSSION

The interaction of granulocyte specific ANF with polymorphonuclear granulocytes in blood smears has been shown in the present study to depend partly on the age of the slides but chiefly on the degree of damage which the leucocytes underwent prior to testing for ANF The three kinds of blood smears used as nuclear antigen unaltered blood smears less than 15 minutes old blood smears altered by repeated freezing and thawing and leucocytes altered mechanically by shaking the blood with glass beads were shown to be nearly equally fitted for the determination of granulocyte specific ANF while unaltered blood smears more than 15 minutes old and leucocytes altered to a lesser degree were found less reactive Thus 6 sera were found which invariably showed no nuclear staining with 60 minute old blood smears indicating that the use of fresh blood smears is important to secure the optimal antigenicity or accessibility ... ocytes

Although the use of fresh non altered leucocytes yielded constant and reproducible results further investigations showed that the degree of alteration which the leucocytes underwent before testing for ANF played an important role in determining the immunoglobulin classes

of ANF reacting with granulocytes therefore this is also of significance for the estimation of the frequency of ANF in human sera While sera with low molecular weight (Ig G) ANF reacted with the fresh intact granulocytes as well as with altered granulocytes many more sera proved to contain ANF of high molecular weight when altered leucocytes were used as nuclear antigen Thus only one third of sera with Ig M ANF and only half of sera with Ig A ANF which were found to react with altered granulocytes combined with unaltered 15 minute old granulocytes suggesting that the intact (living) granulocytes are inaccessible to most ANF of high molecular weight

The possibility that the non reactivity of old blood smears to certain ANF is caused not by any degradation of the nuclear antigen but by an increasing inaccessibility of the leucocytes with age is suggested by the fact that the reactivity of old blood smears could be restored to some extent by freezing and thawing the smears and by the finding that it was always the same 6 out of 20 sera which did not react with leucocytes in 60 minute old blood smears There is however no direct evidence that the lack of reactivity of old smears is related to the molecular size of the antibody since some sera even of Ig G specificity did not react with 60 minute old unaltered blood smears It is however conceivable that the ANF in these 6 sera which did not react with old blood smears formed some kind of high molecular weight complexes *e g* with rheumatoid factors (15)

Inaccessibility of cells to antibodies has been demonstrated before in a few cases The nuclei of sperms have been shown not to react with ANF as the nuclear membrane is not accessible to molecules with a molecular weight more than 60 000 (14) which is considerably under the molecular weight of even 7 S antibodies Since mechanical alteration of leucocytes prior to testing for Le cell factors in human sera caused a considerable increase in the sensitivity of the test (17) this might indicate a similar inaccessibility of unaltered leucocytes to Le cell factors The procedure of freezing and thawing used in the present studies to disrupt the cells is identical with the method employed when tissues are made accessible to high molecular weight complexes of antibodies conjugated with ferritin (5) When working with ferritin labelled antibodies it has been necessary to freeze and thaw the tissue 3 times to obtain maximum accessibility this is in keeping with the result obtained in the present study

The finding that high molecular weight granulocyte specific ANF chiefly reacted with altered granulocytes may probably explain the wide variation in the frequency of *granulocyte reactive ANF* found previously in rheumatoid arthritis (Review see 7) The use of altered granulocytes as nuclear antigen has thus been found to increase the incidence of ANF to 75 per cent as compared with only 30 per cent reacting with intact fresh leucocytes (7) Since ANF in rheumatoid arthritis are mostly high molecular weight antibodies (2 4) this in

dicates that the increased frequency of ANF found in rheumatoid arthritis with the use of altered granulocytes is caused by ANF of high molecular weight

SUMMARY

The interaction of 20 sera containing granulocyte specific ANF with polymorphonuclear granulocytes of various ages and degrees of alteration has been investigated

The use of 15 minute-old unaltered granulocytes was shown to give reproducible results while the use of older leucocytes invariably rendered some sera negative probably owing to an increasing inaccessibility of leucocytes with age since alteration of the cell by repeated freezing restored the reactivity of old blood smears to some extent

In further investigations of the inaccessibility of granulocytes to some ANF it was shown that while all sera with ANF of low molecular weight (Ig G) reacted with unaltered fresh granulocytes only one third of sera with Ig M ANF and only half of sera with Ig A ANF combined with unaltered granulocytes this suggest that the living intact granulocytes are inaccessible to most granulocyte specific ANF of high molecular weight To obtain maximum accessibility it was found necessary to alter the cells by repeated freezing and thawing

This finding may probably explain the wide variation in the frequency of *granulocyte reactive ANF* previously found in sera from patients with rheumatoid arthritis since alteration of granulocytes increases the incidence of ANF in rheumatoid arthritis considerably and since ANF in rheumatoid arthritis are mostly high molecular weight antibodies

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Acta path. et microbiol. scandinav. 69: 393-403, 1967

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HISTOCHEMICAL APPLICATION OF RUTHENIUM RED IN THE STUDY OF MAST CELL ULTRASTRUCTURE

By

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Received 26.viii.66

It is now generally agreed that the metachromatic substance in mast cell granules is heparin. This concept is based upon a relationship between mast cell and heparin content of tissues (*Jorpes et al.* 1937) as well as determination of heparin content of isolated mast cells (*Schiller & Dorfman* 1959, *Bloom & Ringertz* 1960) and isolated mast cell granules (*Parekh & Glick* 1962, *Lagunoff et al.* 1964). While the behavior of mast cell histamine during many pathological conditions is well documented, the fate of granule heparin is more obscure (*Riley* 1959, *Bloom* 1965). Recently it has been shown that rat mast cell granules contain 316 µg/mg dry weight heparin and that the removal of heparin from isolated granules by salt extractions gives no recognizable granule structure left (*Lagunoff et al.* 1964). As heparin obviously constitutes such an integral part of the granule there is some reason for methods making it possible to follow changes in mast cell heparin in anaphylactic as well as cytotoxic reactions.

Recently *Luft* (1964) reported that the inorganic dye Ruthenium red $Ru_2(OH)_2Cl_4 \cdot 7NH_3 \cdot 3H_2O$, early used to stain pectin substances, binds to and precipitates several animal acid mucopolysaccharides. Luft also found that Ruthenium red is sufficiently electron dense to be localized ultrastructurally.

These properties of Ruthenium red led us to investigate the possibility of developing a method using this stain for visualizing the mast cell heparin in light and electron microscopy. Considering the possibility that the acid mucopolysaccharide staining properties of Ruthenium red may be related to its many ammonium groups it seemed reasonable to expect that precipitation of the saccharide polyanions with long chain aliphatic ammonium salts (*Scott* 1960) would interfere

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Presented in abbreviated form at the Annual Meeting of the Scandinavian Society for Electron Microscopy in Gothenburg, Sweden, June 2-3, 1966 (*Gustafson & Pihl* 1966).

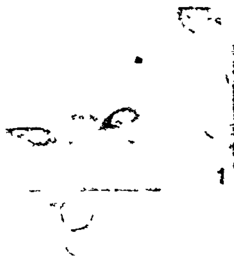


Fig 1

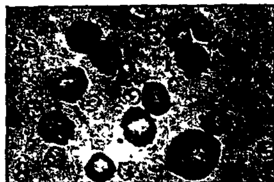
Tissue mast cell from hamster cheek pouch vital stained in 0 05 per cent Ruthenium red fixed in glutaraldehyde and sectioned in a cryostat microtome × 800

Fig 2

Rat peritoneal mast cells treated with cetylpyridinium chloride before staining with Ruthenium red and fixation in glutaraldehyde Evidently, staining of the granules of mast cells is blocked by the quaternary ammonium salt The corresponding result in the electron microscope is illustrated in Fig 12 × 800

Fig 3

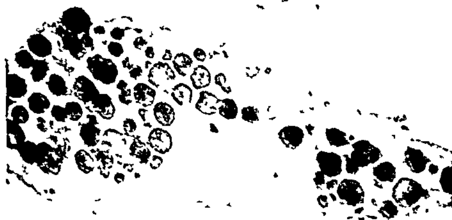
Rat peritoneal mast cells treated as described in Fig 2 but in addition stained red with ferric thiocyanate × 800



Figs 4 5

Fig 4 Tissue mast cell from hamster cheek pouch Fixation in glutaraldehyde containing 0 05 per cent Ruthenium red Post fixation in osmium tetroxide All mast cell granules are of almost equal electron density contrasting to the pale nucleus Uncontrasted as all subsequent figures × 9 700

Fig 5 Detail from the same mast cell as in Fig 4 the granules of which are very electron dense with a fine fibrillar mesh × 14 000



4

5

with a successive staining with Ruthenium red Consequently an attempt was also made to asses the selectivity of the dye for the granule heparin by blocking with cethylpyridinium chloride

MATERIALS AND METHODS

Mast cells were isolated from the peritoneal fluid of 20 male and female Sprague-Dawley rats weighing 200–300 g In pilot studies the following procedure to obtain intact mast cells was experienced as the most convenient Five ml of a 0 1 M phosphate buffer, pH 7 4 containing 7 5 per cent sucrose was injected into the peritoneal cavity of animals anaesthetized with ether Heparin was avoided as it might interfere with the staining In some experiments we used a Krebs Ringer solution without calcium (143 mM NaCl 4 7 mM KCl 1 2 mM $MgSO_4$ 3 1 mM phosphate buffer, final pH 7 2), containing in addition sodium citrate at a concentration of 15 5 mM After gentle massage for 1 5 min the abdomen was opened and the fluid withdrawn An equal amount of a 4° C 0 1 per cent solution of Ruthenium red in the aforementioned phosphate buffer was immediately added The specimen tube was placed in an ice bath for 5 min and centrifuged at 30 × g for 10 min at 4° C The cell sediment was fixed for one hour in 3 per cent phosphate buffered glutaraldehyde at 4° C centrifuged at 300 × g rinsed in 0 1 M phosphate buffer dehydrated and embedded in Epon 812 according to *Luft* (1961) In some cases the cell suspensions were in addition postfixed in Millonig s phosphate buffered OsO_4 for one hour (*Millonig* 1962) The temperature was not allowed to rise above 4° C until infiltration with the epoxy resin

In addition tissue mast cells were studied in hamsters the cheek pouches of which are rich in mast cells and easy of access (*Gustafson & Gronberg* 1963) The animals weighing 80–110 g were anaesthetized with ether and the cheek pouches exposed to 0 05 per cent Ruthenium red in a 3 per cent phosphate buffered glutaraldehyde solution At the same time 2 ml of the same solution was injected intracardially until the animal was killed The cheek pouches were excised cut in thin slices and placed in the same stain fixative solution for one hour at 4° C and washed for another hour in the phosphate buffer Further treatment for electron microscopy was carried out as described for rat peritoneal cells In addition 10 μ thick sections were cut with a cryostat microtome and without further treatment mounted in polysorbitol

From the Epon blocks 1 μ thick sections for light microscopy as well as ultrathin ones were cut with an LKB ultramicrotome Generally contrasting was avoided The ultrathin sections were examined in a Siemens Elmiskop IA at 60 kV

Control and Selectivity Testing Procedures

A paired series of sections corresponding to those above were processed in the same fashion but without using Ruthenium red In order to block the Ruthenium red staining some specimens were exposed to a 0 05 per cent solution of cetylpyridinium chloride (1 hexadecylpyridinium chloride Merck AG Darmstadt Germany) in 0 05 M tris maleate buffer containing 8 72 per cent sucrose and in one series also 3 9 per cent NaCl in order to increase ionic concentration (*Zugibe & Fink* 1966 b)

Figs 6 7

Fig 6 Rat peritoneal mast cell vital stained in 0 05 per cent Ruthenium red and fixed in glutaraldehyde There is a considerable difference in electron density between the granules The distribution of the various types is rather even all over the cytoplasm × 11 000

Fig 7 Detail of a rat peritoneal mast cell treated as for Fig 6 The electron dense granules are rather homogeneous and often smaller than the lighter ones which show a fine fibrillar mesh To the right part of the picture the cell nucleus is seen × 43 000

6

7

8

9

Some of the cell suspensions were then exposed to Ruthenium red and treated as described above for light and electron microscopy. Other cell suspensions exposed to cetylpyridinium chloride were dehydrated, embedded, cut in 1 μ thick sections and exposed to a solution of 0.05 per cent ferric thiocyanate Fe(SCN)$_3$ (*Zugibe & Fink* 1966 a) in 0.05 M tris maleate buffer containing 7 per cent sucrose. The use of tris-maleate buffer was found necessary as ferric thiocyanate in the concentration used was found insoluble in phosphate buffers with corresponding ionic strength.

RESULTS

After vital staining the tissue mast cells appeared intensely red in the cryostat microtome sections. In contrast, other structures were unaffected or had, at most, got only a faint tinge (Fig. 1). It was evident that the selective staining of mast cell granules with Ruthenium red corresponded with the metachromasia produced by toluidine blue solutions. Corresponding results were obtained with 1 μ thick Epon sections of the peritoneal mast cells, the granules of which during the whole process had retained a strong Ruthenium red stain.

In the electron microscope equivalent results were obtained (Figs. 4 and 5). While nuclei as well as other cell organelles and cytoplasm were of low electron density in the uncontrasted sections, the mast cell granules in comparison were markedly electron dense, indicating a selective binding to the latter. When post fixation in osmium tetroxide was avoided the considerable variation in electron density between different granules is striking (Figs. 6 and 7). The least electron dense granules were often large and similar to each other in structure, presenting a fine granular meshwork. The more electron dense granules were more varying in size and showed patches of electron dense material against a homogeneous background.

In contrast, granules only treated with glutaraldehyde varied little in electron density (Fig. 8), as was also the case if post fixation in osmium tetroxide was carried out in addition (Figs. 9 and 10). With the latter technique membranes were distinct while Ruthenium red alone merely stained the contents of the granules leaving membranes hardly discernible.

In cells treated with the Ruthenium red method and post fixed in osmium tetroxide membrane structures were distinct and the dark patches resulting from the Ruthenium red recognizable (Fig. 11). Thus

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Figs. 8–9

Fig. 8. Unstained rat peritoneal mast cell fixed in glutaraldehyde. There is little difference in electron density between different granules and between these and the cell nucleus and adjacent lymphocytes. × 11,200

Fig. 9. Rat peritoneal mast cell treated as for Fig. 8 and in addition post fixed in osmium tetroxide. The mast cell granules are of about equal electron density but obviously the contrast between granules and cytoplasm as well as cell nucleus is enhanced. There is practically no halo around the granules. A few mitochondria can be seen. × 10[illegible]0

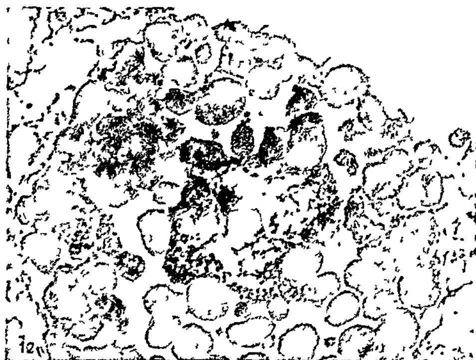


Fig. 12

This peritoneal mast cell was treated with cethylpyridinium chloride before staining with Ruthenium red. Fixation in glutaraldehyde, no post fixation. Cell nucleus and cytoplasm are severely affected by the quaternary ammonium compounds (no NaCl was added). Only a few granules to the left of the cell nucleus show some electron dense traces ascribable to Ruthenium red, while the other granules are very pale although they do not seem to have increased very much in size. × 14 400. This figure should be compared with Figs. 6 and 7.

biological state of different granules. If this is the case, granules of normal mast cells may represent a rather heterogenous population from a functional point of view.

Obviously, there are disadvantages of using a vital Ruthenium red staining in electron microscopy. These are incomplete reproduction of membranes and nuclei and a tendency of mast cells to attract small contaminating particles. We think, however, that the staining of granule heparin makes it possible to follow granule changes during pathological conditions. If this is the main interest, the mast cells ought to be vital stained with Ruthenium red. For such purposes post fixation in osmium tetroxide and/or contrasting should be avoided.

SUMMARY

A method has been developed using the inorganic dye Ruthenium red in the study of mast cells for light and electron microscopy. Ruthenium red was found to react selectively with mast cell granules resulting in

a stable red complex In the electron microscope equivalent results were obtained granules being intensely electron dense After vital staining with Ruthenium red followed by glutaraldehyde fixation mast cell granules were found to present great variations in electron density By blocking the granule heparin with cethylpyridinium chloride the staining with Ruthenium red was inhibited It is concluded that Ruthenium red stains mast cell granule heparin

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Acta path. et microbiol. scandinav. 69: 404–416, 1967

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COMPLEMENT-FIXING LIVER ANTIBODIES

2. *Occurrence in a Material of Patients with Chronic Hepatitis*

By

G. Norup

Received 1.viii.66

In recent years the serology of acute and chronic hepatitis has been the object of several investigations. Before 1957 these were mainly undertaken in the search for a specific serologic test for the diagnosis of acute viral hepatitis. Since then, the investigations have chiefly been concerned with the serology of chronic hepatitis as the detection of liver-autoantibodies in the sera of such patients seemed to provide an explanation of the pathogenesis of the disease.

Based on experiences with the complement fixation test earlier investigators have suggested that a liver damage arisen during the acute phase of infectious hepatitis might evoke a production of antibodies directed against the patient's own liver, thus bringing about the development of chronic hepatitis (*Eaton et al.* 1944, *Bjørneboe & Krag* 1947, *Gear* 1955).

During the course of investigations aimed at detecting infectious hepatitis virus in livers from patients who had died from acute yellow atrophy of the liver, *Gajdusek* (1958) observed a complement fixation with suspensions of liver tissue as antigen in sera from 6 out of 75 (8 per cent) patients with acute viral hepatitis and from 22 out of 33 (67 per cent) patients with various forms of chronic hepatitis. In the latter group of patients complement fixing liver antibodies were demonstrated in 7 out of 9 cases with chronic active hepatitis, in 5 out of 5 cases with lupoid hepatitis – a severe form of chronic active hepatitis associated with positive L.E. cell tests (*MacKay et al.* 1956) – in 7 out of 11 cases of chronic nutritional hepatitis, and in 3 out of 8 cases of biliary cirrhosis. High titres of liver antibodies were found in three of the patients with lupoid hepatitis and in one patient with primary biliary cirrhosis. The complement fixing antibodies were thermostable (60° C for 30 min.), migrated at paper electrophoresis with the slow moving gamma-globulin fraction, and were precipitated from serum with ammonium sulphate. *Gajdusek* therefore regarded them as true antibodies but did not attribute any pathogenic significance to their presence in serum since they were neither organ- nor species-specific.

nor could they be demonstrated in sera from all patients with chronic hepatitis (*Gajdusek* 1958)

This concept of the pathogenic importance of the complement fixing liver antibodies seemed to be further supported by observations reported by *Mackay et al* (1957) and by *Mackay & Larkin* (1958). These investigators found that serum of one case with lupoid hepatitis, one with nutritional cirrhosis, two with lupus erythematodes disseminatus and one with macroglobulinemia failed to react with antigen prepared from the patient's own (autologous) organs and tissues (liver, kidney, spleen and striated muscular tissue), although the same sera gave high titres with antigens prepared from the above mentioned organs and tissues obtained at necropsy from other patients. In a later study (*Mackay & Larkin* 1959) another 10 patients (3 with active chronic hepatitis, 2 with lupoid hepatitis, 1 with nutritional hepatitis, 3 with lupus erythematodes disseminatus and 1 with colitis ulcerosa) whose sera in all cases fixed complement with heterologous organs and tissues, were examined for complement fixing antibodies to antigens prepared from autologous organs and tissues (liver, kidney and muscle). Sera from 3 of these 10 patients failed to react with autologous organs and tissues, while in the remaining 7 cases a positive complement fixation was observed.

The problem of the pathogenic significance of the complement fixing tissue antibodies remains undetermined. Their frequent occurrence in primary biliary cirrhosis and in chronic postviral hepatitis seems, however, to suggest that immunological processes may play a role in the pathogenesis of these types of cirrhosis (*Mackay* 1958, 1960, *Deicher et al* 1960, *Gokcen* 1962, *Pasnick et al* 1962, *Walker et al* 1965). In other forms of cirrhosis where the aetiological agent must be considered known (alcohol, obstruction of the biliary tract owing to calculi, cancer etc.) complement fixing liver antibodies occurred with the same frequency only as in normal individuals (*Mackay & Larkin* 1958, *Gokcen* 1962, *Walker et al* 1965). Consequently, the test for tissue antibodies has been considered of some value in the differentiation between primary biliary cirrhosis and extrahepatic obstructive jaundice (*Gokcen* 1962, *Walker et al* 1965).

The aim of the present study has been to determine the frequency of occurrence of complement fixing tissue antibodies in sera from patients with chronic hepatitis and to assess the diagnostic value of this test.

MATERIAL AND METHODS

The *material* comprised 135 patients with hepatic cirrhosis. In all cases the diagn
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age groups of under 50 the number of men and women was equal. 76 (56 per cent)
of the patients were over 60 years of age. Based on anamnestic data the patient

TABLE 1

Results of Complement Fixation Tests with Liver, Thyroid and Kidney Antigens in Sera of Patients with Chronic Hepatitis and Normal Blood Donors

Organs Used for the Antigen Preparations	135 Patients with Chronic Hepatitis						Normal Blood Donors		
	No of Sera Tested	*No of Sera Positive* Titre		*Percentage* Positive	67 Sera Tested with all Antigen Types		122 Sera Tested with all Antigen Types		
		8 16	≧32		No of Sera Positive	Percentage Positive	No of Sera Positive Titre 8–16	≧32	Percentage Positive
Normal Human Livers (Biopsy)	120	16	27	32.5	19	28.4	5	–	4.1
Normal Livers from Rhesus Monkeys	109	10	17	25.0	18	26.9	5	–	4.1
Normal Human Livers (Necropsy)	92	7	7	15.2	11	16.4	5	–	4.1
Thyrotoxic Human Thyroid Glands (Biopsy)	135	11	8	14.1	10	14.9	5	–	4.1
Normal Human Kidney (Necropsy)	128	17	7	18.8	17	25.4	6	–	4.9

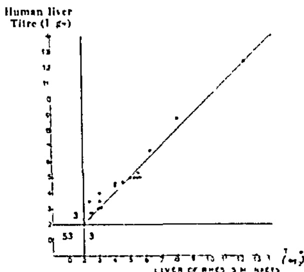


Fig. 3

Relationship between the Titer Values Obtained with Suspensions of Normal Human Biopsy Liver and Normal Liver of Rhesus Monkeys in Sera of 93 Patients with Cirrhosis of the Liver

glands 1) were also positive with liver and/or kidney antigen; thus organ-specific complement fixing thyroid antibodies were demonstrated in 4 cases of chronic hepatitis (only one of these had disease of the thyroid gland). A total of 67 sera from patients with hepatic cirrhosis and 122 sera from normal donors were tested with all the antigen types shown in Table 1. As it appears from this reduced material of patients the frequency of positive tests with antigens from normal biopsy liver, normal liver of rhesus monkeys and normal human kidney tissue obtained at necropsy was almost the same (25.4–28.4 per cent) and higher than that given by antigens from normal human necropsy liver and thyrotoxic thyroid glands (14.9–16.4 per cent), whereas in the donor material a positive reaction was equally frequent (4.1–4.9 per cent) with all the types of antigen.

A total of 93 sera from the patient material was tested with antigens from normal human biopsy liver as well as from normal liver of rhesus monkeys. The results are shown in Fig. 3. A positive complement fixation with the two types of antigen was demonstrated in 26 sera, 66 sera were negative with both antigen types and only one serum was positive with biopsy liver and negative with liver from rhesus monkeys. Normal human biopsy liver and normal liver of rhesus monkeys thus seem to react nearly identically in the complement fixation test.

Fig. 4 shows the incidence of positive reactions with antigens from normal human biopsy liver and/or normal liver from rhesus monkeys in the previously mentioned 4 types of cirrhosis. It will be seen from the

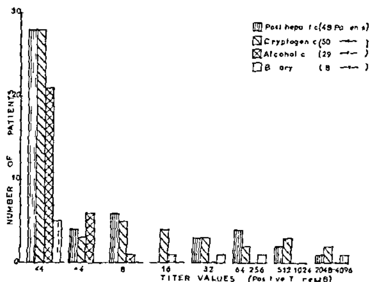


Fig 4

Distribution of Titer Values of Complement Fixing Antibodies to Normal Human Biopsy-Liver and Normal Liver of Rhesus Monkeys in the Various Types of Chronic Hepatitis

figure that positive reactions occurred with the greatest frequency (33–38 per cent) and with the highest titres in the groups of patients with posthepatitic and cryptogenic cirrhosis, and that these two types of cirrhosis differed neither in respect of the frequency of positive tests nor as to the height of the liver antibody titres Of 8 sera from patients with biliary cirrhosis three were positive Of these two sera giving high liver antibody titres were from patients with primary biliary cirrhosis In the group of alcoholic cirrhosis it is noteworthy that only two out of 29 (6 2 per cent) sera fixed complement in the presence of liver tissue in both cases with low titres (8 and 16) and that a relatively large number of sera (6 out of 29) had titres of 4

Table 2 gives the results of complement fixation tests with liver thyroid and kidney antigen on sera from 27 patients with chronic hepatitis It will be seen from the table that all sera except two (Nos 14 and 15), having a high titre (≧64) of liver antibody also reacted positively with antigens from thyroid and/or kidney tissue a result which seems to suggest a certain relationship between high contents of antibody in serum and degree of organ reactivity With the exception of 4 sera (Nos 1–4) which gave high titres with all the antigens listed in the table, there was no relationship between the height of the liver antibody titres and those of the thyroid and kidney antibody titres and no relationship between the results obtained with thyroid and kidney antigens Of 13 sera (Nos 1 13 in Table 2) which fixed complement in the presence of liver, thyroid and/or kidney tissue 10 were from females and 3 from males

TABLE 2

Results of Complement Fixation Test with Antigens from Liver, Thyroid and Kidney Tissue on Sera from 27 Patients with Chronic Hepatitis

Serial No	Age (yr)	Sex	Normal Liver Tissue: Human Biopsy	Normal Liver Tissue: Human Necropsy	Normal Liver Tissue: Rhesus Monkey	Thyrotoxic Thyroid Glands: Human Biopsy	Normal Kidney Tissue: Human Necropsy
1	64	♀	4096	512	4096	2048	512
2	69	♀					
3	65	♀	2048	512	2048	2048	2048
4	57	♀	256	32	256	128	128
5	70	♂	64	8	64	16	8
6	49	♀	32		16	16	8
7		♀	128	8	128	8	
8	[illegible]	♀	128	16	64		8
9	[illegible]	♀	32	8	32		8
10	64	♂					
11	[illegible]	♀	32		32	16	
12	67	♂	32		32		8
13	[illegible]	♀	16		16		16
14	58	♀	1024	128	1024		
15	37	♀	1023	8	1024		
16	75	♀	32		32		
17	69	♂	16		16		
18	78	♀	8		8		
19	67	♂	8				
20	80	♀				8	
21	69	♀					
22	51	♀					16
23	49	♀					
24	71	♀					
25	60	♀					8
26	76	♂					
27	58	♂					

TABLE 3

Sex and Age Distribution of Positive Reactions with Normal Human Biopsy Liver and Normal Liver from Rhesus Monkeys as Antigen in Sera from 135 Patients with Chronic Hepatitis and from 238 Normal Blood Bank Donors

		135 Patients with Chronic Hepatitis				238 Normal Blood Bank Donors		
Age (yr)	Sex	No of Cases Tested	No of Cases Positive	Expected No of Cases Positive Corresponding to the % of Normal Reactors	Positive %	No of Cases Tested	No of Cases Positive	Positive %
18–40	♂	2				99	2	2.3
	♀	2	1			32	1	
41–50	♂	9	1	0.3	33.3	32	2	3.7
	♀	9	5	0.3		22		
51–70	♂	25	7	1.0	30.9	18		3.8
	♀	56	18	2.1		35	2	
71–90	♂	6			25.0			
	♀	26	8					
	♂	42	8	1.2	29.6	149	4	2.9
	♀	93	32	2.7		89	3	

Table 3 shows the sex and age distribution of positive reactions with antigens from normal biopsy liver and liver of rhesus monkeys in the patient material and in the donor material. The frequency of positive tests was greater in the patient material than in the donor material, a result which is further stressed by a comparison of the number of positive cases found with the number of positive cases expected corresponding to the percentage positive of the donor material. In the patient material a positive test occurred more frequently in females than in males in the age groups 11–50 and 71–90, and with almost the same frequency in either sex in the age group 51–70 years. A rise or fall in the frequency of positive reactions with increasing age in either sex was not found.

Sera from three of the patients with chronic hepatitis were examined for complement fixing antibodies against their own (autologous) liver tissue which in all three cases was obtained surgically. The results are shown in Table 4 which further shows the titres obtained with isologous cirrhotic and isologous normal liver tissue as antigen. In two of the cases (L. Jo. and L. J.) complement fixing antibodies to autologous liver tissue were demonstrated, while one case (K. G.) failed to fix complement in the presence of autologous liver tissue. Complement fixing antibodies to suspensions of isologous cirrhotic and isologous normal liver tissue were demonstrated in sera of all cases except one (case L. J.)—the serum of which failed to fix complement with the liver antigen from case K. G. The small amount of liver tissue available precluded further examination of these varying results.

TABLE 4

Results of C mplen ent Fix ti n Tests n S ra fr m three Pat ents u th Chr n c Hepat t s Using Path l g cal Autol g us an l Is l g s as well as N rmal Is l gous Li er Tissue as Ant gen

Type of Cirrh sis	Serum of Case	Antigens [illegible] K.G.	[illegible]	L.J.	Antigens (Normal) Isologous Biopsy Liver
Post Hepatitic	K.G.	4		512	2048
Cryptogenic	L.J.			12	4096
Primary Biliary	L.J.			12	2 6

I

Several investigators have [illegible] d mplement fixing antibodies to liver tissue [illegible] ra from [illegible] with various forms of chronic hepatitis most r ntly [illegible] t posthepatitic (postviral [illegible] necrotic) and cry t [illegible] chronic hepatitis plasma-cell [illegible] titis lupoid hepat t [illegible] r hosis (*Bjorneboe & Kraj* 1 4

normal liver of rhesus monkeys as antigen A positive complement fixation was also found in 18 8 per cent (24 out of 128) and in 14 1 per cent (19 out of 135) of the sera using normal kidney tissue and thyrotoxic thyroid glands, respectively as antigen

Complement fixing liver antibodies were demonstrated in 33–38 per cent of the sera from patients with posthepatitic and cryptogenic types of cirrhosis, in the alcoholic group a positive test occurred in 7 per cent, a frequency which did not differ essentially from that previously demonstrated in a material of normal blood bank donors

The complement fixing liver antibodies were non species and non-organ specific The 'pattern' of organ reactivity was rather constant for a given patient but varied apparently quite arbitrarily from one patient to the other

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Acta path. et microbiol. scandinav. 69: 417–430, 1967

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EXPERIMENTAL STREPTOCOCCAL NEPHRITIS IN RABBITS

By

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Received 15.viii.66

Early in this century a relationship between streptococcal infections and acute glomerulonephritis was considered (*Ophüls* 1916). In 1952 *Rammelkamp et al.* and in 1953 *Rammelkamp*, *Rammelkamp et al.* and *Wertheim et al.* presented epidemiological evidence for a correlation between certain streptococcal types and nephritis, and they suggested the term nephritogenic streptococci for these types. Type 12 streptococci appears to be the most potent etiological agent in the development of acute glomerulonephritis in humans.

Experimental production of a typical acute glomerulonephritis in animals using β-hemolytic streptococci or preparations thereof has not been successful (*Horn* 1937, *Kobernick* 1952, *Tan* 1964). Although hematuria and sometimes albuminuria and hypertension have been induced in such experiments, the histopathological lesions have always been predominantly of tubular nature. Thus *Reed & Matheson* (1954 a and b) were able to produce a clinical picture of acute nephritis in rabbits using type 12 streptococci, however the disease was largely devoid of the glomerular changes characteristic for acute glomerulonephritis in humans. Similar results have been published by *Kelly & Winn* (1958), *Hinkle et al.* (1960) and *Tan & Kaplan* (1962) using different types of intraperitoneal diffusion chambers containing cultures of β-hemolytic streptococci. The cl[illegible] approach in experimental reproduction of acute glomerulonephritis [illegible] to be the so-called nephrotoxic nephritis which may [illegible] by injection of anti-kidney serum. In such experiment[illegible] by *Masugi* (1933, 34) severe glomerular lesions with [illegible] proliferation and crescent formation could be produced.

Recent preliminary reports by *H[illegible] & J[illegible]nsson* (1964) and *Jonsson & Holm* (1964) showed that it is [illegible] by injecting different types of preparations from type 12 strept[illegible] to induce histopathological [illegible]

We wish to thank Prof. *Ö. Ouchterlony* for his stimulating interest and valuable criticism. The skilful technical assistance of Miss *Harriet Andersson* and Miss *Brit-Marie W[illegible]nterhag* is highly appreciated.

well as clinical signs of glomerulonephritis in rabbits The present report comprises more detailed information on the preparation of the material and the development of the histopathological kidney changes

MATERIALS AND METHODS

The nephritogenic streptococcal strain used was isolated from a patient with acute glomerulonephritis during an outbreak of streptococcal throat infections in Bedale England 1963 The strain was an group A type 12 (M+) streptococcus obtained from the Central Public Health Laboratory, Colindale London

Cultivations The streptococci were grown in an antigen free medium described by *Holm & Falsen* (1966) The cultivations were performed at 37° C using an auto matic titrator for pH adjustment (pH 6 8) and the cells were harvested after sixteen hours The cultures were centrifuged at 1 500×g for 20 minutes at 4° C Three washings of the cells with saline buffered with phosphate M/15 pH 7 2 were performed under the same experimental conditions within one hour Five volumes of the wet bacterial sediment were suspended in 95 volumes of buffered saline and the sus pension was kept at 4° C for two weeks It was shaken once or twice daily and finally filtered through a Göttingen No 6 membrane filter The filtrate was tested for sterili ty This preparation was designated streptococcal 'autolysate" and was found to contain 280 γ protein ml as measured by the method described by *Lowry et al* (1951) using lysozyme as a reference substance

Animals 20 rabbits weighing between 2 5 and 3 0 kg were injected once intra venously with 0 5 ml of type 12 streptococcal 'autolysate The rabbits were sacri ficed at different times after the injection (2 4 6 and 8 hours and 1 3, 6 13 20 and 60 days) A control group consisting of two rabbits was injected once intravenously with 0 5 ml saline All rabbits which were kept in the same animal room in separately ventilated animal boxes were obtained from the same farm

Rabbit sera Bleedings of all rabbits were performed immediately before the injection and at the time of sacrifice

Rabbit kidney antigen Kidneys from healthy rabbits were sliced and washed in phosphate buffered saline pH 7 2 Thirty ml buffered saline were added to 10 g of the rabbit kidney preparation and homogenized in an Ultro Turrax (Janke & Kunkel K G Staufen i Br) at 24000 rpm for 3 minutes during cooling in an ice batch The homogenized preparation was then freeze pressed 5 times in an X press (AB Biox Nacka Sweden) This material was centrifuged at 10000×g for 20 minutes The supernate was pipetted off and frozen at —40° C until used The protein content of this preparation was 10 mg ml as measured by the method of *Lowry et al* (1951) using lysozyme as a reference

Diffusion in gel method The rabbit sera were analysed by the double diffusion in gel method of *Ouchterlony* (1958) as modified by *Holm* (1965) against different concentrations of the rabbit kidney antigen (0 01 10 mg/ml)

Complement fixation method The complement fixation method was performed according to the description by *Kabat & Mayer* (1964) Heat inactivated rabbit kidney antigen was used in the highest concentration that gave no anti complementary effect and analysed against the rabbit sera in different dilutions (1/1 1/320)

DPNase activity was determined according to the technique of *Carlson et al* (1957) Determinations of DPNase inhibitors as well as DPNase antibodies were performed according to a technique described by *Holm & Kaijser* (1966)

Streptolysin O (SlO) activity in streptococcal preparations was determined according to *Kalbak* (1947) and expressed in combining units using a standard anti streptolysin from Statens Seruminstitut Copenhagen

Antistreptolysin O determinations were performed according to the technique of *Kalbak* (1947)

Streptolysin S (SlS) activity was determined by measuring the hemolytic activity on a 2 per cent suspension of rabbit erythrocytes Minute amounts of cholesterol were added to inhibit the hemolytic activity of SlO according to *Howard et al* (1953)

Streptococcal proteinase activity was measured by the milk clotting method described by *Elliott* (1945).

Blood pressure readings. The method used for measuring blood pressure in rabbits was a modification of the method described by *Grant & Rothschild* (1934). A lense (magnification 25 ×) was inserted with an air tight seal in the upper end of a metal tube and at the lower end a piece of condom rubber was fixed. This sealed system was mounted on a microscopic stand and connected by a plastic tube to a mercury manometer and by a T tube to a sphygmomanometer bulb. The rabbit's ear was placed between the condom membrane and a glass slide attached to the stage of the microscope. Light from a microscope lamp was directed through the glass slide. By increasing the air pressure the flat condom protruded and compressed the artery. The pressure readings were taken when the first sign of blood reappeared in the artery. Ten consecutive readings were performed and the average value determined (limit values ± 4 mm Hg from the average value). The blood pressure in the ear artery of untreated rabbits varied between 20–30 mm Hg.

Proteinuria and hematuria. Determination of proteinuria was performed semiquantitatively by the albustix method (Ames Co., Stoke Poges, England) and when positive further controlled with the sulfosalicylic acid method (*Kingsbury et al* 1926). The occultest method (Ames Co., Stoke Poges, England) was used as a screening test for hematuria. In case of positive outcome the urine cast was examined microscopically in a Fuchs-Rosenthal chamber.

Histopathological examinations. At different intervals after the injection two rabbits at a time were sacrificed by bleeding. The kidneys were prepared for the histopathological examinations by cutting 1 mm thick slices which were fixed in Zenker solution. The pieces were embedded in paraffin and after sectioning various staining methods were applied such as hematoxylin eosin, hematoxylin van Gieson, Heidenhain-Mallory (?) and finally in some cases the Willer silver impregnation method. At least three sections from each kidney from every rabbit were examined and only definite repeatedly found histologic changes were registered.

RESULTS

Enzyme activity in different streptococcal preparations. Culture filtrate from the type 12 strain used was found to contain 15 combining units SlO and 1200 DPNase units per ml. Streptococcal proteinase was mostly present in the precursor form and even so in small amounts. SlS activity was not detected in the culture filtrate. Neither SlO, SlS, DPNase nor proteinase was found in the "autolysate" preparation but after concentration some DPNase inhibitory effect was noted corresponding to 20–30 inhibitor units per ml unconcentrated "autolysate" material. Antibodies to these streptococcal products could not be detected in any of the rabbits.

Blood pressure readings. After the injection blood pressure readings were performed at different intervals. From Figs. 1 and 2 it is evident that rabbits injected with "autolysate" from the type 12 (M+) streptococcus developed an extreme hypertension. This started almost immediately after the injection and reached a level as high as 3–4 times the initial value (90 mm Hg) during the following 9 hours. The blood pressure attained its maximum (130 mm Hg) after 4–5 days. Two months after the injection the blood pressure was still high but had decreased to 70–80 mm Hg.

Proteinuria and hematuria. Proteinuria was found in one of the two rabbits sacrificed one day after the injection while the second as well as two other rabbits sacrificed two days later showed no proteinuria

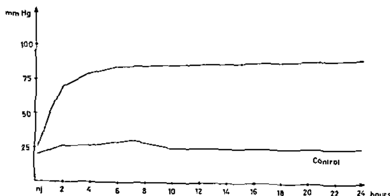


Fig 1

Average blood pressure in the ear artery of twelve rabbits injected with the streptococcal "autolysate" preparation from a type 12 streptococcus (measured by the ear capsule method)

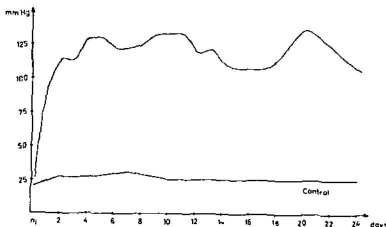


Fig 2

Average blood pressure in the ear artery of four rabbits injected with the streptococcal "autolysate" preparation from a type 12 streptococcus

One of the rabbits, killed 6 days after the injection, as opposed to all of those killed after 13 days, showed the presence of protein in the urine The rabbits sacrificed at later stages had traces to moderate amounts of protein in the urine Hematuria was also occasionally found during the first six days after the injection At later stages half the number of animals developed hematuria, which was often only detected by microscopic examination.

Antibodies against homologous kidney tissue Although three different serological techniques were employed, antibodies against homologous kidney tissue could not be revealed in the sera from any of the rabbits irregardless of the severity of the kidney lesion produced Not even those rabbits which were sacrificed two months after the in-

TABLE 1

Histopathological Changes of the Kidneys

Rabbit number	Sacrificed days	Glomerular changes	Tubular changes
1	1	Intense hyperemia. Enlarged glomeruli	Swelling of epithelium
2	1	Endothelial swelling	Cylinders and red blood corpuscles in the collecting tubules
3	3	0	Solitary red blood corpuscles in the collecting tubes
4	3	Endothelial swelling. Cell proliferation	Red blood corpuscles in the collecting tubes
5	6	Endothelial swelling and endothelial proliferation. Fibrin exsudation in the capsular spaces. Fusion of the capillary loops. Complete destruction of some glomeruli	Granular and hyaline cylinders and red blood corpuscles in tubuli. Cell degeneration
6		Endothelial swelling and endothelial proliferation	Red blood corpuscles in the collecting tubes
	13	Endothelial swelling and endothelial proliferation. Fibrinous exsudation in the capsular spaces. Fusion of the loops. Proliferation of the capsular epithelium	Granular cylinders and lots of red blood corpuscles in tubuli
8	13	Hyperemia. Slight proliferation. Capsular proliferation	Swelling of epithelium
9	20	Endothelial swelling. Fusion of capillary loops	Slight degenerative changes
10	20	Endothelial swelling and endothelial proliferation. Fusion of solitary capillary loops. Proliferation of the capsular epithelium	Granular cylinders and red blood corpuscles in tubuli
11	60	Endothelial proliferation. Fusion of the capillary loops and fibrin exsudation in the capsule. Hemorrhagic necrosis in solitary glomeruli	Granular and hyaline cylinders in tubuli
1	60	Endothelial swelling and endothelial proliferation	Solitary granular cylinders in the collecting tubes

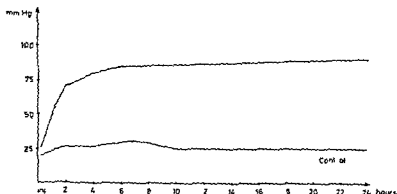


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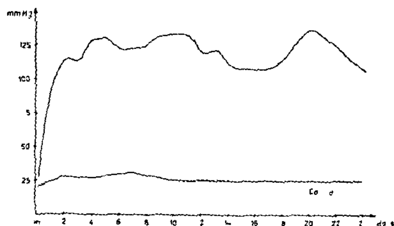


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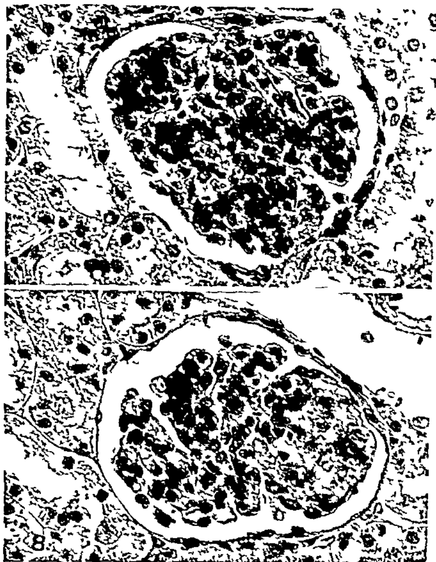


Figs 5–6

Fig 5 Enlarged glomerulus with swollen and proliferating endothelial cells Hematoxylin v Gieson × 400

Fig 6 Two glomeruli one only slightly swollen and with proliferating capillary endothelium the other with advanced changes among other things a fibrinous exsudate in the capsular space Hematoxylin v Gieson × 400

twelve rabbits one (No 3) showed no glomerular changes and one rabbit only an intense hyperemia (No 1) In two animals (Nos 2 and 9) the main glomerular change was a pronounced swelling of the capillary endothelium In three animals (Nos 4, 6 and 12) this swelling was combined with a proliferation of the endothelial cells, especially in rabbit No 6 Even at low magnification the hypercellularity of the damaged glomeruli was striking (Fig 3) In one rabbit (No 10) the

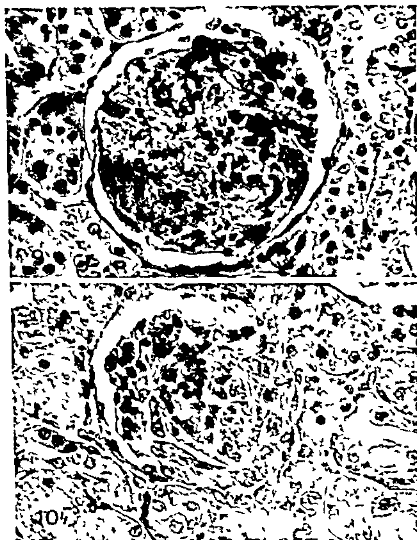


Figs 7 8

Fig 7 Glomerulus with very enlarged endothelium *proliferation of the endothelium* and fusion of the capillary loops Hematoxylin v Gieson × 400

Fig 8 Glomerulus with swollen and proliferating capillary endothelium fused capillary loops and finger like adhesion Hematoxylin v Gieson × 400

capillary loops were fused together and besides the endothelial swelling a proliferation of the capsular epithelium was noted in some glomeruli In three animals (Nos 5 7 and 11) advanced lesions were seen affecting practically all glomeruli Besides swelling and proliferation of the capillary endothelium (Fig 5) fibrinous exsudation in the capsular space (Fig 6) and a finger like fusion of the capillary loops could be seen (Figs 7 and 8) In two of these animals a hemorrhagic necrosis



Figs 9 10

Fig 9 Partially hemorrhagic necrotic gl mer lus Hemat xylin v Gieson × 400
Fig 10 Advanced changes of the gl merulus with s ckle like proliferati n of the epithel um of B wman s cap ul H mat x n v Gieson × 400

of some glomeruli w is also det ct l I ts) In the third rabbit (No 7) a proliferation of the epithel u m in B m an s capsule with development of adhesions betv een the sheaths of th capsule was found (Fig 10) In silver stained sections a net f gr ie e rgentophilic threads could be seen in the changed glomeruli

All rabbits v h h lowed gl m rul r changes also presented tubular lesions These we cc i g nt sw llings of the epithelium in the tubuli contorti ir mbi a r with a decreased affinity for nucl ar

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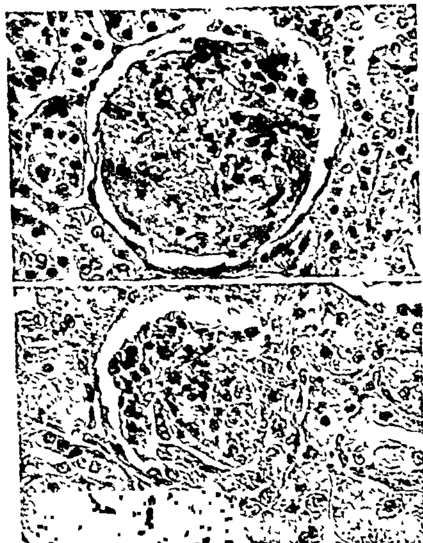
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Figs 9 10

Fig 9 Partially hemorrhagic necrotic glomerulus Hematoxylin v Gieson × 400
Fig 10 Advanced changes of the glomerulus with sickle like proliferation of the epithelium of Bowman's capsule Hematoxylin v Gieson × 400

of some glomeruli was also detected (Fig 9) In the third rabbit (No 7) a proliferation of the epithelium in Bowman's capsule with development of adhesions between the sheaths of the capsule was found (Fig 10) In silver stained sections a net of gracile argentophilic threads could be seen in the changed glomeruli

All rabbits which showed glomerular changes also presented tubular lesions These were seen as gentle swellings of the epithelium in the tubuli contorti in combination with a decreased affinity for nuclear

stains Red blood corpuscles and hyaline and/or granular cylinders especially in the collecting tubes were also noted (Fig 4). In one rabbit (No 3) without glomerular changes, red blood corpuscles were seen in the collecting tubes The severity of the tubular changes was on the whole proportional to the glomerular changes

No histopathological glomerular or tubular changes could be detected in the control rabbits

DISCUSSION

In the preliminary reports on the nephritogenicity of streptococci (*Holm & Jonsson* (1964), *Jonsson & Holm* (1964) it was shown that acute glomerulonephritis could be produced by culture filtrates as well as "autolysate" preparations or penicillin-killed type 12 streptococci These results disagree with the findings of *Reed & Matheson* (1954 b), who could not produce hypertension or any kidney lesions by injections of ten-times washed and killed streptococci This discrepancy can be explained if it is assumed that the nephritogenic factor or factors diffused from the streptococci into the washings and were thus removed from these streptococcal preparations *Tan & Kaplan* (1962) reported that tubular lesions in mice could not be produced by streptococcal (Group A) culture filtrates lacking streptolysin S (SlS) activity and they could not protect the animals against the tubular toxic effect by injection of polyvalent streptococcal antisera On this basis they have suggested SlS as a possible agent responsible for the tubular lesions in acute glomerulonephritis in humans In our "autolysate" preparations no SlS activity was found which may be due to unfavourable conditions for preserving SlS activity during the preparation of the material As was shown in an earlier publication (*Jonsson & Holm* 1964) an "auto lysate" preparation of the streptococcal strain S 84 (Group A, type 3) as well as a similar preparation of a strain of streptococcus fecalis hemo lyticus (Group D) could produce tubular lesions in rabbits but neither glomerular lesions nor hypertension were produced SlS need not there fore be the only nephritogenic agent in acute glomerulonephritis

Furthermore another well-known streptococcal product—streptococcal DPNase—has been considered as a possible etiologic factor in the development of acute glomerulonephritis (*Rammelkamp* 1957, *Bernheimer et al* 1957) In our studies, however we could find no DPNase activity in the autolysate preparation although the corresponding culture filtrate contained 1200 DPNase units/ml The newly described (*Holm & Kaijser* 1966) DPNase inhibitor was present in fairly high amounts and the presence of an inhibited DPNase could therefore not be ruled out On the other hand the strain S 84, as well as many other group A streptococcal strains of different serological types, produce DPNase (*Holm & Kaijser* 1966) which contradicts DPNase as an important nephrotoxic factor, at least in regard to glomerular lesions Furthermore neither SlO nor streptococcal proteinase activity could

be related to nephritogenicity as none of these enzymes could be detected in the autolysate preparation. Nor did the rabbits produce detectable amounts of antibodies against streptolysin O, proteinase or DPNase.

Hypertension was produced within a few minutes after the injection of streptococcal "autolysate" before any demonstrable microscopic glomerular or tubular lesions. The interpretation of these blood pressure findings which were reproducible in all rabbits is difficult. It is of course possible to assume a rapidly progressing kidney damage at this early stage. That at least glomerular changes may develop early is indicated by an extreme hyperemia in the glomeruli of the rabbits sacrificed within 24 hours after the injection. If a glomerular lesion develops immediately after the injection an etiologically important factor would be an ischemia of the kidney. The hypertension could then be comparable to the *Goldblatt* mechanism (1947) with release of renin. It is also possible that the nephritogenic streptococci themselves contain substances which initiate an ischemia of the kidney with secondary release of vaso-excitor material from the kidney. The persistence of the hypertension indicates that a hypertensive factor in nephritogenic streptococci is probably not the *only* etiological agent to the hypertension. It is noteworthy that no latent phase of the development of hypertension was seen after the injection of streptococcal material. The possibility of an antigen antibody reaction as an explanation to the hypertension can of course not be completely ruled out by the experiments performed but the early onset of hypertension would be difficult to explain on immunological bases.

The results of the determinations of hematuria and proteinuria confirm the clinical diagnosis of acute glomerulonephritis. As is also often found in humans, albuminuria was occasionally found in early stages of acute glomerulonephritis. However, in rabbits with advanced histopathological changes (2–3 weeks after the injection) proteinuria was almost always found and often of marked severity. Hematuria was also detected in most rabbits. In fact hematuria seemed to be an early sign of nephritis since blood in the urine was found in some rabbits already the day after the injection. In one of these cases red blood corpuscles and granular cylinders were also found in the tubuli at the histopathological examination. In the tubuli of rabbits sacrificed at later stages erythrocytes, hyaline and/or granular cylinders were an almost regular finding.

The kidney changes observed microscopically correspond essentially to those seen in the acute and subacute stages of glomerulonephritis in humans. However, it was noted that only some of the glomeruli were affected when less pronounced renal changes were produced. Furthermore in some animals lesions were also found corresponding largely to focal glomerulonephritis in humans.

In spite of the established hypertension no hypertensive changes of

the vessels could be demonstrated in the kidneys, which may be due to the short duration of the hypertension

In review articles *Rother & Sarre* (1961), *Cruickshank* (1962), *Dixon & Feldman* (1964) and *Paunz* (1965) discussed different pathogenetic mechanisms in the induction of experimental glomerulonephritis That anti-kidney antibodies can be detected in humans with streptococcal nephritis has been reported by many authors (*e g Lange et al* 1949, *Liu & McCrory* 1958) but the significance of such findings is debatable in spite of the demonstration of host γ-globulin (*Mellors et al* 1957) and C′ (*Lachmann et al 1962)* in glomeruli of patients with glomerulonephritis In our experiments no anti-kidney or anti-streptococcal antibodies could be detected in the sera of the diseased rabbits by any of the three immunological techniques used It therefore seems probable that the kidney lesions produced were the results of a direct toxic effect induced by one or more streptococcal factors rather than an anti-kidney antibody reaction However, we cannot rule out the possibility that an immunological mechanism may also be involved This would then be elicited by a mechanism analogous to the one described by *Kay* (1940) due to fixation of streptococcal antigens to the kidney just as streptococcal M-protein has been found by *Kaplan* (1958) to have an unusual affinity to the glomeruli of the kidney

SUMMARY

Type 12 streptococci isolated from a patient with acute glomerulonephritis, were grown in an antigen free medium under stabilized pH conditions The cells were washed and "autolyzed" by storing at 4° C for 2 weeks After sterile filtration the "autolysate" preparation was injected intravenously into rabbits Clinical and histopathological evidence of the production of glomerulonephritis was obtained No kidney antibodies could be detected in the sera from any of the rabbits

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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *KLEBSIELLA PNEUMONIAE* TYPE 2 (B)

By

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Received 15.viii.66

The polysaccharide isolated from *Klebsiella pneumoniae* type 2 (B) was among the first bacterial polysaccharides to be investigated. As early as in 1925 (4) the polysaccharide was isolated and examined and was found to contain glucose and uronic acid. The polysaccharide showed a chemical and immunological relationship with *Pneumococcus* type 2 (1). This cross reaction was later confirmed (2) by demonstration in anti type 2 pneumococcal horse serum but not in anti type 2 pneumococcal rabbit serum.

More recently (6) the chemical investigation of the capsular polysaccharide isolated from *Klebsiella pneumoniae* type 2 (B) showed it to contain glucose, glucuronic acid and rhamnose which also were the monosaccharides demonstrated in the polysaccharide from *Pneumococcus* type 2.

The purpose of this paper will be purification of the polysaccharide isolated from *Klebsiella pneumoniae* type 2 (B) and a chemical investigation of the structure of the acidic capsular polysaccharide. Most of the methods used for this examination have been described in an earlier paper and will not be repeated here (3).

MATERIAL

The crude polysaccharide of *Klebsiella pn*[illegible] *m*[illegible] type [illegible] B strain Amsterdam F 24 was extracted by cold water from a [illegible] agar [illegible] as described earlier (5). The polysaccharide obtained by th[illegible] was further separated by treatment [illegible] n [illegible] acidic and one neutral. The [illegible] fraction. The purification with [illegible] change in the optical rotation of [illegible] rotation was measured in a [illegible] as Na salt and found to be

$\alpha_{D}^{20} = + 3$[illegible]

1 g of crude p[illegible] gave 83 [illegible] of acidic polysaccharide which did not contain nitrogen or phosphorus. The [illegible] capsular polysaccharide isolated from *Klebsiella pneumoniae* type 2 (B) strain Amsterdam F 24 obtained after this purification was used for further chemical investigations.

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EXPERIMENTAL AND RESULTS

Hydrolysis of the Acidic Polysaccharide

The monosaccharide components were determined after hydrolysis with sulphuric acid. The hydrolysate was neutralized with barium carbonate and centrifuged. The supernatant was treated with Amberlite Resin I.R. 120 (H⁺) and concentrated to a small volume ready for paper chromatography. Descending chromatography on Whatman paper no. 1 was used. Solvent developers were

1. Butanol-ethanol-water 4v: 1v: 5v
2. Ethyl acetate-pyridine-water 2v: 2v: 5v
3. Pyridine-ethyl acetate-glacial acetic acid-water 5v: 5v: 1v: 3v
4. Butanol-ethanol-water-ammonia 40v: 10v: 49v: 1v

Most of the chromatograms were run twofold for spraying with the two different reagents a) anilin hydrogen phthalate and b) silver nitrate–sodium hydroxide.

Hydrolysis with Different Concentration of Sulphuric Acid

The same amount of polysaccharide (10 mg per ml of acid) was hydrolysed for 3 hr at 100° with different concentration of sulphuric acid: 2N, 1N, 0.1N and 0.01N. The hydrolysates were neutralized in the usual way and submitted to paper chromatography in solvent 1 and 2. The hydrolysates were found to contain:

2N sulphuric acid: glucose, mannose, uronic acid and aldobiuronic acid.

1N sulphuric acid: glucose, mannose, uronic acid and aldobiuronic acid but also a fraction with R_f value between those for uronic acid and glucose.

0.1N sulphuric acid gave the same fractions as hydrolysis with 1N sulphuric acid but on a smaller scale.

With 0.01N acid most of the polysaccharide was still unhydrolysed but a faint spot of mannose could be seen.

When the polysaccharide was hydrolysed with 2N, 1N or 0.1N sulphuric acid and the paper chromatogram of the hydrolysates was sprayed with silver nitrate-sodium hydroxide, a spot appeared which could not be seen when sprayed with aniline hydrogen phthalate. The R_G value corresponded with the lactone spot of glucuronic acid.

The results from the different hydrolyses showed that the polysaccharide was composed of glucose, a uronic acid which easily formed lactone, a small amount of mannose and an aldobiuronic acid.

Cellulose Column Chromatography of the Hydrolysate of the Polysaccharide

The preliminary hydrolysis indicated that 1N sulphuric acid probably would give a disaccharide different from aldobiuronic acid. This

concentration was therefore used when a big portion of the polysaccharide was hydrolysed for column chromatography.

400 mg of acidic polysaccharide was hydrolysed with 1N sulphuric acid for 3 hr at 100°. The hydrolysate was treated in the usual way before evaporation to dryness to obtain the weight which was 300 mg. The column was packed with Whatman standard grade cellulose powder as a slurry in solvent no. 1, which was used as developer during the chromatography. The hydrolysate was applied to the top of the column dissolved in solvent no. 1. Fractions of 5 ml were collected by an automatic fraction collector at a rate of three fractions an hour. Spots of each second fraction were applied to a paper chromatogram in solvent no. 2 and sprayed with silver nitrate sodium hydroxide. Five different fractions were collected from the column and identified by paper chromatography as glucose, mannose, uronic acid, aldobiuronic acid and another disaccharide with R_F value between those for glucose and uronic acid.

The glucose fraction showed an optical rotation $[\alpha]_D^{20} = +44°$ (c 0.4 water). The glucose was further identified by treatment with glucose oxidase. Gluconic acid was formed which could be seen when the paper chromatogram was sprayed with silver nitrate sodium hydroxide.

Mannose was only identified on paper chromatograms in solvent no. 1, 2 and 3 since the amount isolated was too small to make any derivatives.

The uronic acid was identified as glucuronic acid by conversion to the methyl ester *methyl glycoside, reduction* with *lithium aluminium* hydride, hydrolysis and paper chromatography of the hydrolysate. This contained only glucose, which was further identified by glucose oxidase.

The aldobiuronic acid fraction was hydrolysed with 2N sulphuric *acid for 8 hr and was found to contain glucose and uronic acid*; the disaccharide was converted to the methyl ester methyl glycoside and reduced with lithium aluminium hydride. The hydrolysate of the methyl bioside contained glucose only, which was further identified by glucose oxidase. The aldobiuro[illegible] acid was th[illegible]refore identified as *glucuronic acid linked to glu[illegible]s*[illegible]

The other disaccharide [illegible] had a R_F [illegible] between those for *glucose* and glucuronic [illegible] *hydrol*[illegible] *and* was *found to be* composed of ma[illegible]e and glu[illegible]ose.

Periodate Oxi[illegible] *f the Acidic Polys*[illegible]*c haride*

The acidic [illegible] haride was oxi[illegible] by sodium periodate at room temperature [illegible] The consumption of periodate was analys[illegible] periodically [illegible] results [illegible]re plotted against time and can be seen in Table 1. It se[illegible] as [illegible] oxidation was *complete after 48 hr* and

overoxidation started This was confirmed when the oxidation was repeated at 4°

To see if acid was formed during oxidation, aliquots of the reaction mixture were analysed Excess of periodate was removed by addition of ethylene glycol, and acid formed during the oxidation was titrated against 0 01N sodium hydroxide potentiometrically by means of a glass electrode and a pH-meter The sults can be seen in Table 1.

TABLE 1
Periodate Oxidation of the Polysaccharide

Time in hr	1	4	24	48	72	120	144
Mol periodate per mol anhydrosugar	0 5	0 56	0 91	1 04	1 21	1 40	1 38
Mol formic acid formed per mol anhydrosugar		–	0 30	0 46	0 54	0 54	–

Any measurable amount of formaldehyde was not formed during the oxidation with periodate

The polysaccharide was isolated after oxidation and hydrolysed By paper chromatography it was found to contain mostly glucose Only traces of mannose and glucuronic acid were left

Methylation of the Acidic Polysaccharide

Haworth's method was used for methylation The procedure was repeated four times After isolation, the methylated polysaccharide was hydrolysed in two stages, first by treatment with 5 per cent methanolic hydrogen chloride and then with 2N sulphuric acid The hydrolysate was applied to a paper chromatogram in solvent no 4 The chromatogram was stopped before the solvent front left the paper, about 17 hr Six spots were seen when the paper was sprayed with anilin hydrogen phtalate When the hydrolysate was deionized with De-acidite Resin IF (CO_3) before chromatography, two of the spots disappered (M_g = 0 16 and 0 06) which indicated that these spots belonged to the methylated *uronic acids)* M_g*-value for 2,3,4 6-tetra O-methyl-glucose in the same* solvent was 1 00

By comparing ionophoresis in 0 1M borate buffer, pH — 10 and M_g values by paper chromatography, the neutral methylated monosaccharides have been identified as 2,3 4,6 tetra-O-methyl mannose 2,4,6-tri-O methyl-glucose and 2,3-di O-methyl-glucose One of the spots could not be identified, and could well be due to incompletely methylated sugar

The methylated glucuronic acid was removed from the hydrolysate by De-acidite Resin IF (CO_3) The resin was treated with ammonium carbonate, to get the acid out again as ammonium salt The solution

with the ammonium salt was treated with Amberlite Resin I R 120 (H+) to obtain the methylated uronic acid as free acid. The acid was then converted to methyl ester methyl glycoside, reduced with lithium aluminium hydride, hydrolysed and the methylated glucose was identified as 2,3,4-tri-O-methyl glucose.

By comparing the results obtained after oxidation and after methylation, the glucuronic acid could be linked 1-6. Most of the glucose seemed to be linked 1-3, but some glucose is engaged in branching as 1-4-6 linked.

The mannose is linked to glucose either in 1-6- or in 1-4-position and will probably form the non-reducing end group at the branch point.

DISCUSSION

The first investigation of the capsular polysaccharide isolated from *Klebsiella pneumoniae* type 2 (B) showed it to contain glucose and uronic acid. Later the presence of rhamnose and the nature of the uronic acid as glucuronic acid were determined. This very clearly explained the cross reaction between *Klebsiella pneumoniae* type 2 (B) and *Pneumococcus* type 2, since both the type specific polysaccharides were composed of identical monosaccharides.

However, in the examination of the acidic type specific polysaccharide from *Klebsiella pneumoniae* type 2 (B) which has been referred in this paper, rhamnose could not be demonstrated. Rhamnose was not found neither in the crude polysaccharide nor in the purified acidic polysaccharide. Instead of rhamnose mannose was found. This monosaccharide has not been reported as constituent of the polysaccharide from *Klebsiella pneumoniae* type 2 (B) before. The amount of mannose was very small, in fact too small to isolate for making derivative. The presence of mannose has therefore only been ascertained by paper chromatography, but in three different solvent developers.

The mannose seemed to be the non-reducing end group at the branch point. The presence of 2,3,4,6-tetra-O-methyl mannose after methylation and hydrolysis confirmed mannose to be the non-reducing end group. Mannose was also preferentially released on treatment of the polysaccharide with 0.01N sulphuric acid for 3 hr at 100°.

The polysaccharide cannot have a highly branched structure, since oxidation with periodate did not form any measurable amount of formaldehyde.

Glucose seemed to form the branch point which was proved by identification of 2,3-di-O-methyl glucose. The isolation of a small amount of the disaccharide composed of glucose and mannose confirmed glucose as branch point.

Most of the glucose, however, must be a part of the chain structure and linked 1-3. After methylation and hydrolysis of the polysaccharide most of the glucose was identified as 2,4,6-tri-O-methyl glucose. When

the polysaccharide was isolated and hydrolysed after oxidation, glucose was found in a big yield

The glucuronic acid seemed to be linked 1,6 By oxidation formic acid was formed The identification of 2,3,4-tri-O-methyl-glucose after reduction of the methylated uronic acid fraction, confirmed the presence of 1,6-linked glucuronic acid The uronic acid is linked to glucose as an aldobiuronic acid

The glucuronic acid linked 1,6 could be the main common feature in the two type specific polysaccharides isolated from *Klebsiella pneumoniae* type 2 (B) and *Pneumococcus* type 2, and make the foundation for the cross-reaction between the two species Another point could be the presence of glucose as branch point in both polysaccharides

SUMMARY

1 The acidic type specific polysaccharide of *Klebsiella pneumoniae* type 2 (B) was isolated and further purified for structure analysis
2 The polysaccharide contained glucose and glucuronic acid, but also a small amount of mannose was found
3 The glucuronic acid was linked to glucose as an aldobiuronic acid
4 The glucuronic acid was linked 1,6
5 *Most of the glucose was linked 1,3, but some of the glucose formed* branch points in 4- or 6-position
6 Mannose could be the non-reducing end group at the branch point

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SPECIFICITY OF RHEUMATOID FACTORS IN SERA FROM BLOOD DONORS

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Received 30.viii.66

The rheumatoid factors are usually γM globulins and behave as antibodies to determinants of the Fc fragment of the γG globulin molecule (11). This antibody property is utilized for their detection. In the most frequently employed tests for rheumatoid factors, erythrocytes sensitized by human (isologous) or rabbit (heterologous) γG globulin are used (see 11 and 25). The rheumatoid factors are inhibited by whole γ globulin in native or denatured form.

Rheumatoid factors which detect genetic characters (anti-Gm factors) are most often found in sera from patients with rheumatoid arthritis. They may also result from transfusion (2, 22) or fœto-maternal (5) or materno-foetal (23, 24) immunization. Some sera from healthy individuals without known immunization may contain these types of rheumatoid factors.

Rheumatoid factors which do not detect genetic characters are also found in sera from certain patients and from some healthy individuals. Earlier results showed that in many sera from patients with rheumatoid arthritis the reactivity of these factors was caused by separate molecules with specificity for either human or rabbit γ globulins, besides cross-reacting factors (14, 15, 26, 27).

In the present work a large panel of blood donor sera was tested for rheumatoid factor using rabbit γ globulin (Waaler-Rose test). The reactivity of some of the positive sera with human γ globulin was investigated and the specificity of the factors was compared to that described for sera from patients with rheumatoid arthritis. The incidence of positive reactions in the normal population was not considered since it has been extensively discussed in earlier literature (see 28).

MATERIALS AND METHODS

Sera. Sera from 2985 blood donors were obtained from the Serological Laboratory, Department of Microbiology, the Gade Institute. They had been sent for routine

This investigation was supported in part by U. S. Public Health Service Research Grant 5 RO5 TW 001 ? 01 from the National Institutes of Health.

syphilis serology The blood donors had passed an ordinary clinical examination and showed no signs of disease Some rheumatoid sera used in a previous study (26) were included

The incomplete anti D sera the reference anti Gm sera and the rabbit anti human γ globulin serum and specific anti γG anti γA and anti γM sera were the same as used previously (16) Anti γA and anti γM sera were puchased from Behringwerke AG Marburg-Lahn Germany Incomplete anti CD serum Ripley was kindly provided by Dr *Marion Waller* of the Department of Pathology School of Medicine Richmond Va USA

Rabbit anti sheep erythrocytes serum was prepared as described earlier (25)

The sera were kept frozen at -20° C in small aliquots All sera except the anti D sera were heated for 30 minutes at 56° C before use

Treatment of sera by 2 Mercaptoethanol was performed as described previously (27)

Gamma globulin Human γ globulin (Fraction II) 12 per cent solution was purchased from AB Kabi Stockholm Sweden Rabbit γ globulin (lyophilized Fraction II a preparation of Pentex Inc Kankakee Ill) was a gift from Dr *F Milgrom* Buffalo N Y The protein concentration was determined by a modified Folin method (13) using the γ globulin from AB Kabi as standard

Denatured γ globulin preparations were prepared by heating a 1 per cent solution at 63° C for 10 minutes

Erythrocytes Human erythrocytes (O, R_1R_2) from a single donor were collected in sterile acid citrate dextrose solution Sheep erythrocytes were obtained from whole blood collected in Alsever s solution Before use the erythrocytes were washed 4 times in 10 volumes of saline (phosphate buffered saline pH 7 2) They were finally packed at 1 000 × g for 5 minutes

Tests for anti γ globulin factors The Waaler Rose test was carried out as described elsewhere (25 26) After the tubes had stood overnight at 4° C the agglutination was recorded by gently tapping each tube

The tests with anti D sensitized erythrocytes were performed as previously described using an excess of incomplete anti D antibody for sensitization (16) The sensitized erythrocytes were always tested with rabbit anti γ globulin serum and reference anti Gm sera before use One drop each of serum dilution saline and a one per cent suspension of sensitized erythrocytes were mixed and left at 20° C for 1 hour Before reading the tubes were centrifuged at 1 000 × g for 30 seconds

The latex (RA) test was performed as recommended by the manufacturer with reagents purchased from the Hyland Laboratories Los Angeles Calif USA

Inhibition of agglutination The γ globulin preparations were properly absorbed by unsensitized or by sensitized erythrocytes in order to remove agglutinating activities against unsensitized and sensitized erythrocytes Absorbed γ globulin was tested for inhibiting activity by adding 0 1 ml of a 0 5 per cent solution to 0 1 ml of twofold dilutions of the serum to be tested After incubation for 1 hour at 20 C the sensitized erythrocytes were added and agglutination was recorded in the usual manner

Absorption of anti γ globulin factors Absorption using human γ globulin was performed by mixing 0 2 ml of serum dilution 1 in 2 and an equal volume of packed erythrocytes sensitized by anti CD Ripley in excess The mixture was left at 20° C for two hours The supernate (absorbed serum) was removed after centrifugation

Absorption using rabbit γ globulin was performed by mixing 0 2 ml of serum diluted 1 in 8 and packed sheep erythrocytes sensitized by ten agglutinating units of rabbit antiserum The mixture was left at 4° C overnight The absorbed serum was tested against unsensitized and sensitized sheep erythrocytes

Preparative ultracentrifugation Preparative ultracentrifugation was performed according to the method described by *Kunkel et al* (10) using 16-hour separation in a 10 40 per cent sucrose gradient Ten successive 0 5 ml fractions of the effluent were obtained from a pin hole in the bottom of the tubes

EXPERIMENTS AND RESULTS

Agglutination Experiments

Of the 2285 blood donor sera 2137 (93.5 per cent) gave titres of 16 or less in the Waaler Rose test (Table 1). Such weak reactions were considered as negative. Titres of 32 or 64 were obtained with 130 of the sera (5.7 per cent), and were regarded as doubtful positive reactions. Titres of 128 or higher obtained with 18 sera (0.8 per cent), were considered as definite positive reactions. The latter group comprised an equal number of males and females aged between 20 and 55.

Because of the small amounts of each serum available only 14 sera were further studied. Twelve of these were selected among those which gave highest titres in the Waaler Rose test. Two sera which gave no reaction in the Waaler Rose test were also included.

None of the sera agglutinated erythrocytes sensitized with anti D specific for Gm(1), Gm(4) or Gm(5) respectively. The results obtained in the anti CD Ripley test and in the latex test are shown in Table 2. The latex test positive sera gave either positive or negative reactions in the anti CD Ripley test while all the latex test negative sera gave no reaction in the anti CD Ripley test. One of the Waaler Rose test negative sera (No 1010) was positive in the anti CD Ripley test and the latex test.

TABLE 1

Distribution of the 2285 Blood Donor Sera According to Titres in the Waaler Rose Test

	Titre in Waaler Rose test					
	≦16	32	64	128	256	Total
Number of sera	2137	88	42	15	3	2285
Per cent of sera	93.5	5.7		0.8		100

TABLE 2

Fourteen Selected Blood Donor Sera Grouped According to Titres in the Waaler Rose Test and to Results with the Latex and the Anti CD Ripley Tests

		Titre in Waaler Rose test			
		≦16	32-64	≧128	Total
Latex test positive	Anti CD Ripley test positive (titre ≧4)	1010	865 994	8 579	5
	Anti CD Ripley test negative (titre <4)		849 972	267 470 586	5
Latex test negative	Anti CD Ripley test positive				0
	Anti CD Ripley test negative	468	760	2 6 56a	4

Absorption Experiments

The specificities of the anti-γ-globulin factors involved in the reaction with erythrocytes sensitized by human and rabbit antibodies were tested in absorption experiments with six of the sera The results with two blood donor sera, and one rheumatoid arthritis serum included as control, are shown in Table 3 Sheep erythrocytes sensitized with rabbit γ-globulin easily removed the homologous activity, while the activity detected by the other test system was hardly influenced Similarly, human erythrocytes sensitized with human γ-globulin removed only the homologous activity This was found with the monospecific serum (849), showing reaction only in the Waaler-Rose test, as well as with the polyspecific serum (994) which reacted in both tests Accordingly, anti-γ-globulin factors in sera from some blood donors exhibited a strict specificity, comparable to those in sera from patients with rheumatoid arthritis (14, 26) The other four sera contained mainly cross-reacting factors

TABLE 3

Titres in Anti CD Ripley and Waaler Rose Tests of two Blood Donor Sera (NHS) and one Rheumatoid Serum (RAS) Effect of Absorption with Erythrocytes Sensitized by Rabbit and Human γ Globulin

Serum absorbed with	Anti CD Ripley test NHS 994	Anti CD Ripley test NHS 849	Anti CD Ripley test RAS 4429	Waaler-Rose test NHS 994	Waaler-Rose test NHS 849	Waaler-Rose test RAS 4429
Sheep erythrocytes						
sensitized	8	<2	32	<8	<8	8
unsensitized	8	<2	32	128	64	256
Human erythrocytes						
sensitized	<2	<2	<2	128	64	256
unsensitized	16	<2	32	128	64	256

Controls
Unsensitized erythrocytes and respective sera diluted 1 in 2 no agglutination
Sensitized erythrocytes and saline no agglutination

Inhibition Experiments

The inhibiting effect of native and denatured human and rabbit γ globulin on the agglutination in the Waaler-Rose and the anti CD Ripley tests was investigated using the same sera as above

Rabbit γ-globulin, either native or denatured, inhibited only the reaction in the Waaler Rose test, while the reaction in the anti-CD Ripley test was not influenced (Table 4) Human γ-globulin inhibited completely the reaction in the anti CD Ripley test, while the reaction in the Waaler-Rose test was only partially inhibited Denatured human γ-globulin gave somewhat stronger inhibition than native γ-globulin

With one blood donor serum, 849, which reacted in the Waaler-Rose test only there was no inhibition with either of the human γ-globulin preparations

TABLE 4

Effect of Native and Denatured Human or Rabbit γ-Globulin on Titres of two Blood Donor Sera (NHS) and one Rheumatoid Serum (RAS)

Test in	Anti-CD Ripley test NHS 994	NHS 849	RAS 4179	Waaler-Rose test NHS 994	NHS 849	RAS 4179
Saline	16	2	32	128	128	256
γ-globulin (5 mg/ml)						
rabbit native	16	<2	32	<8	<8	<8
„ denatured	8	<2	32	<8	<8	<8
human native	<2	<2	<2	32	64	32
„ *denatured*	<2	<2	<2	16	64	16

Controls: Unsensitized erythrocytes and saline: no agglutination.
Sensitized erythrocytes and saline or the respective γ-globulin preparations: no agglutination.

The findings were in accordance with those obtained in absorption experiments and with previous studies on rheumatoid factors in sera from patients with rheumatoid arthritis (14, 26). Different specificity of rheumatoid factors in normal human sera seems to depend on separate factors with specificity for human or rabbit γ-globulin or for both species γ-globulins.

Physicochemical Properties

By preparative ultracentrifugation of three blood donor sera (579, 849, 994) the hetero- and isospecific rheumatoid factor activity was found in the bottom fractions. These fractions contained γM-globulin as shown in test with specific anti-γG, anti-γA and anti-γM sera. The rheumatoid factor activity was also inactivated by mercaptoethanol. Accordingly, the serological activity was attributable to the γM-globulin.

DISCUSSION

Rheumatoid factors which detect genetic characters show a high degree of specificity (11). More than 20 such anti-Gm factors are known at present (12, 18, 21). It is also known that anti-Gm factors from normal individuals (Snagg factors) show a higher degree of specificity than anti-Gm factors from patients with rheumatoid arthritis (Ragg factors). Ragg anti-Gm factors are inhibited by large amounts of sera which are negative for the given Gm factor while Snagg factors are not. Furthermore, Snagg factors are inhibited by very small amounts of serum positive for a given factor.

The anti-Gm factors are usually isospecific and do not react with γ-globulin from other species (11).

In contrast, the specificities of rheumatoid factors which do not detect genetic character are much discussed (see 26). Some authors

have claimed that the reactivity with γG-globulin of different species is only due to cross-reacting factors (1, 4) However, accumulating evidence shows that rheumatoid factors with species-restricted specificity do exist as well as cross-reacting factors (14, 15, 26, 29)

In the present study, results of agglutination, absorption and inhibition experiments indicated that rheumatoid factors in sera from blood donors behaved in a similar way Separate factors reacting with rabbit γ globulin only, with human γ-globulin only, or with both, were demonstrable by absorption experiments In inhibition experiments even heat denatured γG-globulin only partly inhibited the activity against rabbit γ-globulin (the Waaler-Rose test) with some of the sera

Heat denaturation mainly changes sites on the Fc fragment with which rheumatoid factors react, causing rupture of disulphide bonds and intermolecular bridging (7) Antigen-antibody reaction on the other hand, mainly causes changes of the Fab fragment of the molecule (8 17) It is thus reasonable that heat denatured γ-globulin will be more prone to give cross-reactions than γ-globulin altered in an antigen-antibody complex like an agglutinate Rheumatoid factors may, however, also react with heat denatured Fab fragments (9)

Various findings further indicate that the antibody is more extensively changed in precipitation reactions than in agglutination *Aho & Simons* (1) observed that immune precipitates containing γG-globulin as antibody were able to absorb all rheumatoid factors present in a given serum Even precipitates with Gm(1) γG-globulin absorbed an anti-Gm(1) factor By this procedure rheumatoid factor was also absorbed using autologous γG-globulin

A corresponding finding was made with anti-γ-globulin factors detected by pepsin digested γ-globulin Such factors were not inhibited by undigested native γ-globulin and were not absorbed by red cells sensitized by whole anti-D In contrast, immune precipitates containing whole γG antibodies removed the activity (6) Obviously then, the reactivity of various rheumatoid factors and other anti-γ-globulin factors depends on the state of the γ-globulin used for their detection

Various recent findings indicate that autospecific rheumatoid factors, *i e* factors which react with the individual's own native γG-globulin exist besides the isospecific and heterospecific factors (3, 11, 20 27) Such factors may complex with the autologous γ-globulin *in vivo* and cannot be demonstrated in whole serum (3) Anti-γ-globulin factors detected by pepsin-digested γG-globulin (16, 19) and probably also anti-antibodies (see 19), show primary specificity for autologous γG globulin

The present results clearly show that rheumatoid factors in normal human sera possess similar specificities as were earlier shown with rheumatoid arthritis sera (14, 15 26, 29) These findings indicate that the rheumatoid factors express only a quantitative and not a qualitative deviation from the normal state (25)

SUMMARY

S ra from 228 blood d nors were titrated in the Waaler Rose test Of these sera 93 per cent gave no reaction 5 7 per cent a doubtful reaction and 0 8 per cent showed a definite positive reaction

Fourteen of the positive sera were selected for more extensive studies including the anti CD Ripley and the latex tests

Agglutination absorption and inhibition experiments indicated that the reactions with human and rabbit γ globulin were produced by two independent factors besides cross reacting factors Accordingly rheumatoid factors in normal human serum and in rheumatoid arthritis serum showed similarly restricted specificities Only quantitative and not qualitative differences seemed to exist between rheumatoid factors in the two types of sera

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Acta path. et microbiol. scandinav. 69: 445–457, 1967

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CORRELATION OF DNA BASE COMPOSITION AND ACID FORMATION FROM GLUCOSE OF STAPHYLOCOCCI AND MICROCOCCI

By

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Received 13.ix.66

Classification of gram-positive, catalase-positive cocci has been a point of controversy since the early days of bacteriology.

At times all these organisms were placed in only one genus — named either *Micrococcus* or *Staphylococcus* — at other times they were placed in two different genera, *Micrococcus* and *Staphylococcus*.

The Subcommittee on Taxonomy of Staphylococci and Micrococci (1965a) has recognized two genera, *Micrococcus* and *Staphylococcus*, and proposed to separate staphylococci from micrococci by use of the ability of staphylococci to grow and produce acid from glucose when incubated under anaerobic conditions.

DNA base composition clearly divides gram-positive, catalase-positive cocci into only two groups: one having a high percentage of guanine + cytosine (65–75 per cent) and one having a low percentage (30–36 per cent). Studies by *Silvestri & Hill* (1965) and by *Boháček et al.* (1965) indicate that strains with high per cent GC values are micrococci and strains with low per cent GC values are staphylococci. *Silvestri & Hill* identified their strains as micrococci and staphylococci on the basis of a taxometric analysis. *Boháček* and his colleagues distinguished the two kinds of strains on the basis of the ability to produce acid from glucose anaerobically.

The main purpose of the pres[illegible] an analysis of the correlation between DNA base comp[illegible] the ability to produce acid from glucose among gram-po[illegible] positive cocci. Thirty different strains on which DNA [illegible] had been made by the above mentioned authors were coll[illegible] subjected to a number of different tests including potent[illegible] pH determinations of aerobic and anaerobic cultures in a m[illegible] containing glucose.

MATERIAL AND METHODS

Origin of Strains and per cent GC Data

30 strains of gram positive and catalase positive cocci were received from the National Collection of Type Cultures (NCTC), London and from the Czechoslovak Collection of Microorganisms (CCM), Brno (Table 1)

TABLE 1
List of Strains Used

Name	Coll number	Other number or names
Micrococcus		
luteus	CCM 132	
	CCM 840	
	CCM 1674	
„	CCM 337	*Sarcina lutea* ATCC 382
	CCM 169	*M lysodeikticus* ATCC 4698
„	CCM 855	*S afermentans* NCTC 2665 NCIB 9278 Hill 40
roseus	CCM 679	ATCC 186 NCTC 7523 NCDO 975 Hill 41
	CCM 837	ATCC 416 NCTC 7518
„	CCM 385	ATCC 185 NCTC 7515 NCIB 9149
	CCM 560	ATCC 179 NCTC 7514
„	CCM 633	ATCC 412 NCTC 7517 NCIB 8175
spp	CCM 740	
	CCM 836	
„	CCM 2087	
„	CCM 825	
Staphylococcus		
afermentans	NCTC 7563	Hill 48
roseus	NCTC 7511	ATCC 144 NCDO 974 Hill 38
„	NCTC 7512	ATCC 177 CCM 557 Hill 39
aureus	NCTC 4136	Hill 1
„	NCTC 4163	Hill 2
„	NCTC 6571	ATCC 9144 Oxford H NCDO 907 Hill 3
	NCTC 8532	ATCC 12600 NCDO 949 Hill 4
	CCM 529	
epidermidis	CCM 901	*Micrococcus cerolyticus* ATCC 12559
saprophyticus	NCTC 7292	NCIB 8711 NCDO 948 Hill 21
„	NCTC 7612	NCDO 783 Hill 23
lactis	NCTC 189	Hill 29
„	NCTC 7564	Hill 31
	NCTC 7944	Hill 32
	CCM 1400	

CCM Czechoslovak Collection of Microorganisms
NCTC National Collection of Type Cultures
ATCC American Type Culture Collection
NCIB National Collection of Industrial Bacteria
NCDO National Collection of Dairy Organisms
Hill Strain no used by *Hill* (1959) *Silvestri & Hill* (1965) *Hill et al* (1965)

The data on per cent GC values of the NCTC strains were drawn from the results of *Silvestri & Hill* (1965). The per cent GC values of the CCM strains were communicated by *Boháček et al* (1966)

The strains were kept on nutrient agar plates (medium A) with the following composition beef heart 500 g peptone 10 g NaCl 3 g Na_2HPO_4 (12 H_2O) 2 g tap water 1 000 ml agar 1 7-2 0 per cent All test media were inoculated from overnight cultures grown at 35° C on this medium

Morphol gy and m tility were determined by micr sc[illegible] of an overnight plate culture incubated at 35° C suspended in tap water Gram staining was performed by the following method crystal violet, 1 per cent aqueous solution 1 minute, Lugol's iodine solution 1 minute ethanol 96 per cent ½ minute Counterstaining with carbol fuchsin

Acid Production from Carbohydrates

A) *In fluid medium* Ability to produce acid from glucose aerobically and anaerobically in a fluid medium was tested electrometrically with "pH meter PHM 22 k (Radiometer, Copenhagen) The following medium was used trypticase (BBL) 1 0 per cent, yeast extract (Difco) 0.5 per cent, NaCl 0 5 per cent, K_2HPO_4 0 1 per cent, glucose 1 0 per cent distilled water (*Evans et al* 1955) Cultures were grown in cotton plugged 150 × 13 mm test tubes with 5 ml medium Anaerobic conditions were obtained by use of an evacuated anaerobic jar (Baird & Tatlock London Ltd) The last trace of oxygen was removed by addition of an appropriate volume of hydrogen after evacuation After incubation at 35° C, readings were made on the second fourth and the sixth day Control tubes containing the same medium without glucose were inoculated and pH was measured after anaerobic incubation for 6 days Uninoculated control media were also examined

[illegible]

pH 7.2 (Subcommittee 1958b) For determination of anaerobic production of acid from glucose and mannitol the medium was dispensed in an 80 mm layer After inoculation the medium was covered with a 25 mm layer of sterile paraffin oil A change of colour from purple to yellow throughout the tube within 6 days was recorded positive In the test for aerobic acid formation from glucose mannitol galactose lactose maltose and mannose the medium was dispensed in layers 25 mm high The tubes were inoculated by stabbing to the bottom of the tube Incubation was at 35° C, and readings were made daily for 6 days

Other Tests

Arginine reaction This test was carried out by two methods

(I) As a growth experiment in the following medium peptone (Orthana special) 0 5 per cent beef extract (Lab Lemco) 0 5 per cent bromcresol purple (1 500) [illegible] pyridoxal 0 5 per cent, glucose 0 05 [illegible] per cent distilled water Adjusted

[illegible] n bromocresol purple (1 500) 0 5 per cent cresol red (1 500), 0.25 per cent L(+) arginine monohydrochloride 1 0 per cent, 1/15 M K_2HPO_4, 1 1 per cent 1/15 M KH_2PO_4 8 9 per cent, distilled water

Control media of (I) and (II) were prepared without arginine The media were dispensed in cotton plugged tubes 155 × 9 mm both with and without a cover of a 25 mm layer of sterile paraffin Medium (I) was inoculated lightly with a straight inoculating needle Medium (II) was inoculated by pipette with 0 2 ml of a suspension made from three loopfuls of a fresh nutrient agar culture in 1 ml distilled water Incubated at 35° C with uninoculated controls Readings after 2 and 4 hours and daily for 5 days Development of a la nder c lour was recorded as positive All controls remained unchanged in colour

Catalase A small amount of plate [illegible] was deposited by means of a glass rod in a drop of 3 per cent H_2O_2 on a slide [illegible] of gas bubbles was read as positive

Coagulase The production of free coagulase was tested by heavy inoculation of cotton plugged tubes 155 × 9 mm prepared as follows horse blood is drawn to a final volume of 200 ml in bottles containing 20 ml of a 10 per cent sodium citrate solution Separation of plasma from blood corpuscles is achieved by standing for 24 hours Dispensed in tubes in 3 ml amounts and incubated at 35° C. Read after 2 4 and 18 hours Definite clot formation was recorded positive

Deoxiribonuclease A nutrient agar medium (medium A composition given above with 0 2 per cent of the sodium salt of thymus gland deoxiribonucleic acid (BDH) was streak inoculated and incubated for 40 hours at 35° C Plates were then flooded

with 1 N HCl and clearing of the medium around the areas of growth was recorded as positive (*Jacobs et al* 1964)

Gelatin hydrolysis and H_2S formation Observed by stab inoculation of 155 × 9 mm tubes sealed with paraffin treated corks with the following medium Liebig's meat extract 0 75 per cent peptone (Parke Davis), 2 5 per cent, NaCl 0.5 per cent gelatin 12 per cent, $FeCl_3$ (4 H_2O), 10 per cent Incubation at 22° C and inspected daily for 14 days

Haemolysis Haemolysis was observed on nutrient agar plates (medium A composition given above) containing 5 per cent citrated horse blood The appearance of a clear zone around single colonies after incubation at 35° C observed daily for 3 days was considered indication of haemolysis

Nitrate reduction A medium with 0 02 per cent nitrite free KNO_3 and 0 5 per cent peptone (Bacto) was inoculated and incubated at 35° C for 20 hours The presence of nitrite was detected by adding five drops of 0 8 per cent sulphanilic acid in 5 N acetic acid and five drops of 0 5 per cent α naphthylamine in 5 N acetic acid A red colour indicated the presence of nitrite The presence of unreduced nitrate was checked by the addition of a small amount of metallic zinc dust Occurrence of a red colour indicated that nitrate was still present

Oxidase Tetramethyl para phenylendiamine dihydrochloride (1 per cent) in distilled water was soaked on to filter paper Culture from a nutrient agar plate was smeared on the impregnated paper The development of a strong blue colour within 10 seconds was registered as positive (*Kovacs* 1956)

Phosphatase Production of phosphatase was tested in the following medium peptone (Orthana special) 1 25 per cent, beef extract (Lab Lemco) 0 75 per cent NaCl 0 5 per cent agar (Japan) 1 2 per cent, 10 0 per cent $CaCl_2$ ($2H_2O$) 0 02 per cent distilled water and the sodium salt of phenolphthalein diphosphate in a final concentration of 0 01 per cent (*Barber & Kuper* 1951) Streak inoculated plates were incubated at 35° C for 18 hours Free phenolphthalein was detected by placing a few drops of an ammonia solution (about 25 per cent NH_3) in the lid of each Petri dish The development of a bright pink colour was recorded as positive

Pigmentation Pigment formation was examined in plate cultures incubated for 20 hours at 35° C and then for 3 days at room temperature (20–22° C) The following medium was used peptone (Difco) 0 5 per cent yeast extract (Difco) 0 5 per cent glucose 1 0 per cent agar 2 0 per cent pH adjusted to 7 2

Splitting of Tween 80 The presence of a Tween 80 splitting enzyme was demonstrated by spot inoculation of peptone agar plates containing peptone (Difco) 1 0 per cent NaCl 0 5 per cent $CaCl_2$ (2 H_2O) 0 01 per cent agar (Bacto) 1 5 per cent Tween 80 1 0 per cent Activity was indicated by the presence of an opacity around the culture after 18 hours incubation at 35° C and a further incubation at room temperature for 4 hours (*Sierra* 1957)

Urease A urea splitting enzyme was demonstrated by two methods (a) by inoculation of a medium of the following composition peptone (Bacto) 0 1 per cent NaCl 0 5 per cent K_2HPO_4 0 2 per cent urea 1 0 per cent phenol red (1 500) 0 6 per cent Distilled water Incubation at 35° C Daily readings for 6 days A red colour indicated a positive reaction (b) By making a heavy suspension of culture in the following solution K_2HPO_4 0 1 per cent KH_2PO_4 0 1 per cent NaCl 0 5 per cent phenol red (1 500) 0 1 per cent urea 1 0 per cent Control solution without urea Readings after 20 minutes 1 hour and 24 hours A red colour indicated a positive reaction All controls remained unchanged in colour

Voges Proskauer reaction A medium containing peptone 0 5 per cent K_2HPO_4 0 5 per cent glucose 0 5 per cent and distilled water was inoculated and incubated at 30° and 35° C A sufficient number of tubes were inoculated to permit testing for acetoin production after 2 and 5 days incubation Tubes were placed in a slanting position in the incubator The test for acetoin was made by adding to the culture 1 ml of a 6 per cent alcoholic solution of α naphthol and 0 4 ml of 40 per cent KOH A red colour developed within 1 hour indicated the presence of acetoin

RESULTS

All strains (Table 1) were found to be non motile, gram positive and catalase-positive spheres in pairs and clusters

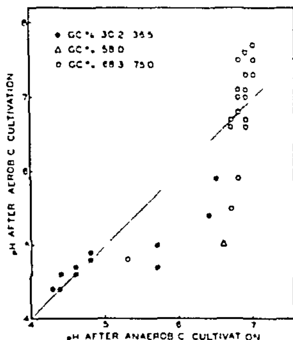


Fig 1

pH values of cultures grown in a glucose medium for six days under anaerobic and aerobic conditions

Acid production from glucose under aerobic and anaerobic conditions as indicated by the pH of the cultures on the sixth day is shown in Fig. 1 and Table 2 The data of deoxyribonucleic acid (DNA) base composition are also given as reported by the examiners (Table 2)

In Fig 1 the pH values of each strain are plotted in a diagram to show the relationship between the aerobic and the anaerobic pH values after cultivation for 6 days By using separate symbols for strains with high and low per cent GC values the relationship between the pH values and the GC values is shown in the same diagram

In Table 2 the same values are tabulated and here the strains are divided into three sections provisionally designated A B and C by differences in their production of acid from glucose Within each section the strains are arranged according to the aerobic pH values starting with the strains showing the lowest value

Section A contains 7 strains all with low per cent GC values (31 2 36 5) and all showing pH values bel[illegible] both aerobically and anaerobically This level of acidification is reached already on the second day of cultivation These strains were all received as *Staphylococcus* and 5 of them as *Staphylococcus aureus*

Section C includes 1[illegible] strains all with high per cent GC values (68 3 75 per cent) and all having pH values greater than 6 5, both aerobically

TABLE 2

pH Values of Staphylococ and Micrococ Cultures

Name and number of strains	Per cent GC values	pH values Incubation six days: glucose medium, anaerobic, average (range)	glucose medium, anaerobic, No of readings	glucose medium, aerobic	basal medium, anaerobic	Section
Staphylococcus aureus CCM 529	34 2	4 3 (4 2 4 4)	2	4 4	7 2	A
Staphylococcus epidermidis CCM 901	35 6	4 4 (4 3 4 4)	2	4 4	7 2	
Staphylococcus lactis NCTC 7944	36 5	4 4 (4 3 4 4)	2	4 6	7 1	
Staphylococcus aureus NCTC 4136	32 8	4 6 (4 6 4 6)	2	4 7	7 2	
Staphylococcus aureus NCTC 8532	31 2	4 6 (4 5 4 7)	2	4 6	7 1	
Staphylococcus aureus NCTC 6571	32 0	4 8 (4 7 4 9)	5	4 8	7 1	
Staphylococcus aureus NCTC 4163	31 2	4 8 (4 7 4 8)	2	4 9	7 3	
Staphylococcus lactis NCTC 7564	69 0	5 3 (5 0 6 3)	5	4 8	7 4	B
Staphylococcus saprophyticus NCTC 7292	31 6	5 7 (5 5 6 0)	6	4 7	7 1	
Staphylococcus saprophyticus NCTC 7612	30 8	5 7 (5 3 6 1)	7	5 0	7 1	
Staphylococcus lactis CCM 1400	30 2	6 4 (6 4 6 5)	3	5 4	7 1	
Staphylococcus lactis NCTC 189	32 8	6 5 (6 4 6 8)	3	5 9	7 2	
Micrococcus sp CCM 740	58 0	6 6 (6 5 6 7)	2	5 0	7 1	
Micrococcus sp CCM 2097	68 8	6 7 (6 6–6 9)	3	5 5	7 6	
Micrococcus sp CCM 825	69 5	6 8 (6 7 6 9)	5	5 9	7 7	
Micrococcus sp CCM 836	68 3	6 7 (6 6 6 8)	3	6 6	7 1	C
Micrococcus roseus CCM 837	63 7	6 7 (6 7 6 7)	2	6 7	7 1	
Micrococcus roseus CCM 679	69 0	6 8 (6 8 6 8)	2	6 8	7 0	
Staphylococcus roseus NCTC 7512	72 8	6 8 (6 7 6 9)	2	7 0	7 1	
Staphylococcus roseus NCTC 7511	75 0	6 8 (6 6 6 8)	5	7 1	7 2	
Staphylococcus afermentans NCTC 7563	72 8	6 8 (6 6–7 0)	5	7 5	7 2	
Micrococcus luteus CCM 132	71 0	6 9 (6 9 6 9)	2	6 6	7 1	
Micrococcus roseus CCM 560	71 0	6 9 (6 9 6 9)	3	6 7	7 1	
Micrococcus luteus CCM 385	71 0	6 9 (6 9–6 9)	2	7 0	7 1	
Micrococcus roseus CCM 633	72 8	6 9 (6 9 6 9)	2	7 1	7 1	
Micrococcus luteus CCM 337	72 3	6 9 (6 8 7 0)	2	7 3	7 0	
Micrococcus luteus CCM 1674	71 5	6 9 (6 9 7 0)	5	7 6	7 3	
Micrococcus luteus CCM 855	73 0	7 0 (6 9 7 0)	2	7 3	7 1	
Micrococcus luteus CCM 169	71 0	7 0 (6 9 7 1)	2	7 5	7 1	
Micrococcus luteus CCM 840	71 0	7 0 (6 9 7 0)	5	7 7	7 0	
Uninoculated control		6 8 (6 7 6 8)	2	6 8	7 0	

and anaerobically, on the second, fourth and sixth day of cultivation 12 of these strains were received as *Micrococcus* and 3 as *Staphylococcus*

Section B consists of 8 strains and is not homogenous either in pH values or in per cent GC values. Four strains have low GC values and the 4 other strains have high values. The pH values are intermediate between the values of the two other sections. It was observed that the pH values fell gradually throughout the observation period. This is in contrast to the results with the strains in sections A and C, where the pH values reached their final value after only 2 days' incubation and did not change essentially on further incubation. Five of the strains of section B were received as *Staphylococcus* and 3 as *Micrococcus*

The accuracy of the pH determination by our method has been tested by calculation of the variance

$$\left(s^2 = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}\right)$$

within strains of the results from cultures grown anaerobically for 6 days. Results of sections A and C strains showed a total variance of 0.0075 (f=40). Section B strains on the other hand displayed considerable inhomogeneity of results. The variance of section B was 0.09 (f=26), mainly due to the results of strains NCTC 7564 (variance 0.30, f=4), NCTC 7612 (variance 0.12, f=6) and NCTC 189 (variance 0.05, f=2). The cause of the large variance values of section B is not clear.

The results of the other tests performed on the strains are shown in Tables 3 and 4. Table 3 shows the results of all strains with low per cent GC values and includes the whole of section A and 4 strains of section B. Table 4 shows the results of all high per cent GC strains and includes the whole of section C together with the 4 strains of section B having high GC values.

It can be seen that the reaction patterns of the strains in Table 3 divide the strains into four small groups numbered 1 to 4 in the table. This subdivision seems to correlate with the final pH value in an anaerobic glucose culture. However, the differences are in some instances very small.

All the strains in Table 3 have low GC per cent values. The ability to metabolize arginine is found in all strains with low GC per cent values and is the only character which is not found in any of the strains having a high GC value (Table 4).

Fermentative production of acid from glucose as determined by indicator changes is common to the strains of section A.

The 4 strains of section B in Table 3 either very in this respect (NCTC 7292) or only produce colour change in the top of the medium and therefore cannot be considered as true fermenters of glucose.

The strains in Table 4 all have high GC values. There is no unique

TABLE
Biochemical Characteristics of

Strains	pH in fluid glucose medium Incub. 6 days anaer (ave.)	aer	Per cent GC values	Acid in semi anaerobic: glucose	mannitol
Staphylococcus aureus NCTC 8532	4 6	4 6	31 ?	+	+
Staphylococcus aureus NCTC 6571	4 8	4 8	32 0	+	+
Staphylococcus aureus NCTC 4136	4 6	4 7	32 8	+	+
Staphylococcus aureus NCTC 4163	4 8	4 9	31 ?	+	
Staphylococcus aureus CCM 529	4 3	4 4	34 2	+	+
Staphylococcus lactis NCTC 7944	4 4	4 6	36 5	+	+
Staphylococcus epidermidis CCM 901	4 4	4 4	35 6	+	—
Staphylococcus saprophyticus NCTC 7292	5 7	4 7	31 6	or +	or —
Staphylococcus saprophyticus NCTC 7612	5 7	5 0	30 8		or —
Staphylococcus lactis CCM 1400	6 4	5 4	30 2		
Staphylococcus lactis NCTC 189	6 5	5 9	32 8		—

\+ = positive — = negative = weak reaction n top of the medium × = positive
No H_2S formation was observed All strains hydrolysed urea by the meth is

character common to all strains and the reaction patterns do not allow of a subdivision of the strains into minor uniform groups The results in the indicator tubes with glucose show that the majority of the strains do not produce acid if they do the reaction is of the oxidative type However the first strain in the table (NCTC 7564) must be characterized as a varying fermenter of glucose

DISCUSSION

The purpose of our experiments was to evaluate and contrast two criteria recently proposed for division of the *Staphylococcus Micrococcus* group of bacteria viz the anaerobic formation of acid from glucose and DNA base composition (per cent GC values)

The data on DNA base determination of the *Staphylococcus Micrococcus* group so far published (*Silvestri & Hill* 1965 *Bohaček et al* 1965 and others) appeared very valuable as a basis of separation by showing a clear cut bimodal distribution of the GC per cent values These were found to be either low (30 36 per cent GC) or high (65 75 per cent GC) The significance of the odd DNA base ratio of strain CCM 740 (*Bohaček et al* 1966) requires further study

Evans et al (1955) examined acid production from glucose by elec

Strains with ... per cent GC

	Production in ... medium																
		aerobic															
	mannitol	galactose	lactose	maltose	mannose	Nitrate reduction	Arginine reaction	Tween 80 splitting	Gelatin hydrolysis	Coagulase	Desoxyribonuclease	Phosphatase	Acetoin	Orange pigmentation	Haemolysis	Section	Group
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—		
		—	+	+	+	+	+	+	+	—	+	+	+			A	1
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
	+	—	+	+	+	+	—	+	+	+	—	+	+	+	+		
	+	+	+	—	—	+	+	+	+			—			—		
	+	+	+	—	+	+	+	+	+		—					A	2
	—	+	+	+	+	+	+	+	+		—	—					
+	—	—	+	+	—	—	×			—	—	—	+		—	B	3
+	—	+	+	+	—	—	×	—	—	—	—	—	+		—		
		—	—	—	—	+	×	—		—	—	—			—	B	4
		—		—		+	×	—		—		—			+		

when done as rapid test negative as growth test All strains were oxidase negative
— the presence of paraffin oil cover did not influence the results

trometric pH determination of *Staphylococcus—Micrococcus* cultures Their data of pH values indicated a bimodal distribution of strains After anaerobic cultivation 4 of their cultures had pH values either equal to or lower than 5 1 or equal to or higher than 6 5 Aerobic cultivation similarly indicated a bimodal distribution 6 strains having pH values equal to or less than 5 5 and the rest equal to or above 6 9 units

Bohaček et al (1965) reported agreement between the DNA base ratio of staphylococci and micrococci and the anaerobic production of acid from glucose determined colorimetrically in a semi solid medium with bromocresol purple as indicator In their study 6 strains fermented glucose and had per cent GC values from 30 2 to 35 6 Eighteen of their strains failed to produce acid from glucose anaerobically and had per cent GC values of 65 5 to 75 5

The results of this study do not show the same clear-cut bimodal distributions of strains

The pH values of cultures grown anaerobically and aerobically in a glucose medium reveal a tendency to ... around either high or low pH values but a number of strains with intermediary pH values were also found

The strains of the present study were tentatively grouped in three sections by the following criteria

TABLE
Biochemical Characteristics of

Strains		pH in fluid glucose medium Incub 6 days anaerobic (average)	aerobic	Per cent GC values
Staphylococcus lactis	NCTC 7564	5 3	4 8	69 0
Micrococcus sp	CCM 740	6 6	5 0	58 0
Micrococcus sp	CCM 2097	6 7	5 5	68 8
Micrococcus sp	CCM 825	6 8	5 9	69 5
Micrococcus sp	CCM 836	6 7	6 6	68 3
Micrococcus luteus	CCM 132	6 9	6 6	71 0
Micrococcus roseus	CCM 837	6 7	6 7	69 7
Micrococcus roseus	CCM 560	6 9	6 7	71 0
Micrococcus roseus	CCM 679	6 8	6 8	69 0
Staphylococcus roseus	NCTC 7512	6 8	7 0	72 8
Micrococcus roseus	CCM 385	6 9	7 0	71 0
Staphylococcus roseus	NCTC 7511	6 8	7 1	75 0
Micrococcus roseus	CCM 633	6 9	7 1	72 8
Micrococcus luteus	CCM 337	6 9	7 3	72 3
Micrococcus luteus	CCM 855	7 0	7 3	73 0
Staphylococcus afermentans	NCTC 7563	6 8	7 5	72 8
Micrococcus luteus	CCM 169	7 0	7 5	71 0
Micrococcus luteus	CCM 1674	6 9	7 6	71 5
Micrococcus luteus	CCM 840	7 0	7 7	71 0

+ = positive, — = negative, = weak reaction on top of the medium No strains pro
or anaerobically The following tests were negative coagulase phosphatase acetoin H-S
arginine test under aerobic conditions when done as growth test (method I), otherwise all

Section A is characterized by strong and fast acid production under anaerobic conditions The strains all have low GC per cent values

Section C is composed of strains with high GC per cent values and no capacity, or only a slight one, for aerobic or anaerobic production of acid

Section B strains form small amounts of acid from glucose under aerobic conditions, and anaerobic formation of acid is slow and weak It includes strains with both low and high GC per cent values

The different levels of acidification in a glucose medium probably reflect differences in the method of glucose metabolism, and these differences might be of greater classificatory value than determination of pH levels However, a determination of these characteristics was outside the scope of our study

The biochemical characteristics of the 11 strains with low per cent GC values (Table 3) suggest the presence of four groups, although the small number of strains in each group do not permit definite recommendations The 7 strains of section A seem divided into two groups by

t

19 Strains with High per cent GC

Acid production in semi solid medium: anaerobic: glucose	aerobic: glucose	aerobic: galactose	Nitrate reduction	Gelatin hydrolysis	Urease	Oxidase	Haemolysis	Pigment	Section
or +	+	+	—	—	+	—	—	yellow	
	+	—	—	+	—	—	—	orange	B
			+	+	+	—	—	yellow	
	+	—	+	—	+	—	—	yellow	
	+	—	+	+	—	—	—	orange	
—	—	—	+	—	—	—	—	yellow	
—	—	—	+	—	—	—	—	pink	
—	—	—	—	—	—	—	—	pink	
—	—	—	+	—	—	—	—	pink	
—	—	—	+	—	—	—	—	*pink*	
—	—	—	+	—	+	—	—	pink	
—	—	—	+	—	—	—	—	pink	C
—	—	—	+	—		—	—	pink	
—	—	—	—	+	+	—	—	yellow	
—	—	—	—	—	+	+	—	yellow	
—	—	—	—	—	—	+	+	yellow	
—	—	—	—	—	+	+	—	yellow	
—	—	—	—	+	+	—	+	yellow	
—	—	—	—	—	—	+	—	white	

duced acid from mannitol mannose, maltose and lactose in semi solid medium aerobically deoxyribonuclease and Tween 80 splitting Strain CCM 1674 was positive repeatedly in the strains were negative in this test in both ways Both urease tests gave similar results

the coagulase, phosphatase and acetoin tests and also by a difference in GC per cent value level within the low GC per cent group

This indicates that minor differences in GC values within the complex having low GC values may be a useful guide for the internal subdivision by biochemical characters

The 4 section B strains with low GC per cent values seem divisible by differences in aerobic acid production of some carbohydrates and by the nitrate and acetoin tests

The strains with high GC per cent values (Table 4) appear inhomogeneous by the tests employed in this study and no internal subdivision is suggested by the results

A positive arginine reaction was common to all low GC per cent strains, in contrast to the negative reaction with the high GC level strains This is the only character *found fully correlating with the* DNA base ratio

A division of the *Staphylococcus Micrococcus* group according to DNA base ratio seems in principle the most acceptable one, especially

in view of the fact that two widely separated levels appear to exist

We find that the proposal of the Subcommittee (1965a) to separate staphylococci from micrococci on the basis of their fermentative glucose catabolism fails to fulfil the necessary condition for its application, i e the presence of a clear-cut bimodal distribution in acid production from glucose in these bacteria However, the test proposed by this Subcommittee may in most cases provide a convenient method in routine work

The taxonomic position of the slow and late acid producers from glucose (section B strains) would be clear by accepting DNA base determination results as a basis for identification of such strains, but the laborious procedure by present methods prevents its application in routine work The arginine reaction could be a useful substitute if proven to be fully correlated with the DNA base ratio determination in a large material of strains

SUMMARY AND CONCLUSION

Gram-positive, catalase-positive cocci differ quantitatively in their anaerobic production of acid from glucose when determined electrometrically

The differences in production of acid under anaerobic conditions do not permit a clear-cut separation in two groups and thus cannot be correlated with the bimodal distribution of per cent GC values of the strains

Differences in both speed and strength of production of acid during anaerobic and aerobic cultivation do suggest a division by pH values of the strains in this study in three sections one section of strong and fast producers of acid from glucose, all with low per cent GC (staphylococci), one section with little, if any formation of acid from glucose all strains with high per cent GC values (micrococci), one intermediate section with strains of both high and low GC values which slowly produces minor amounts of acid from glucose under anaerobic or aerobic conditions

Biochemical characters of the strains with low per cent GC values indicate a possible non-arbitrary subdivision by per cent GC values anaerobic and aerobic formation of acid from glucose, possession of coagulase and phosphatase, capacity of gelatin hydrolysis and splitting of Tween 80

High per cent GC strains appear inhomogeneous in biochemical characters

Among a number of biochemical tests, a positive arginine test was the only biochemical test fully correlating with a DNA base ratio of 30 2 36 5 per cent guanine + cytosine

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Acta path et microbiol scandinav 69, 458–464 1967

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SEROLOGIC INVESTIGATIONS ON TEICHOIC ACIDS FROM THE WALLS OF *STAPHYLOCOCCUS EPIDERMIDIS* AND *MICROCOCCUS*

By

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Received 23 viii 66

The teichoic acid in the wall of *Staphylococcus aureus* is of the ribitol type with alpha- or beta-linked N-acetylglucosaminyl residues It has been shown by agglutination inhibition (13) and by agar gel precipitation (6, 3) that the configuration of the glycosidic linkage determines the serologic specificity Thus two antigenic activities are found in the wall teichoic acids of *Staph aureus*

In *Staph epidermidis* the wall teichoic acid is of the glycerol type with alpha- or beta-glucosyl residues (2) The serologic specificity on agar gel precipitation of a preparation containing glucosyl glycerol teichoic acid isolated from a *Staph epidermidis* strain was demonstrated by *Losnegard & Oeding* (11) This antigen was considered to be identical with carbohydrate B described by *Julianelle & Wieghard* (10) *Morse* (12) isolated a glycerol teichoic acid containing glucose from another strain of *Staph epidermidis* (Prengel) The preparation gave a precipitation line on agar gel against homologous antiserum and precipitin inhibition studies suggested that the antigenic determinant was alpha linked glucose Preliminary serologic studies on some *Staph epidermidis* teichoic acid preparations have been reported in (2)

The *Micrococcus* group seems to comprise strains which have either ribitol, glycerol or no teichoic acid in their walls (1)

In the present paper the antigenic activity of wall teichoic acids from some strains of *Staph epidermidis* and micrococci have been studied

MATERIAL AND METHODS

Teichoic acid was isolated from the walls of five *Staph epidermidis* strains and two micrococcal strains as previously described (1 The teichoic acids are shown in Table 1 (see also (2 From two of the strains (T1 T2 also other extracts were prepared (see below The following purified preparations were used as references in the serologic investigations polysaccharide A (beta glucosaminyl *ribitol* teichoic acid (5 and polysaccharide 263 (alpha glucosaminyl ribitol teichoic acid

(9) isolated from *Staph aureus* strains Wood 46 and 263 KSF C respectively, polysaccharide 1234 (10) (glucosyl glycerol teichoic acid) isolated from *Staph epidermidis* strain 1234 (11) and protein A isolated from *Staph aureus* strain Cowan I (4). These preparations produce specific precipitation lines in agar gel in high dilutions when diffused against their homologous antibodies. The bacteria used in the agar diffusion experiments were live 18 hr cultures on nutrient agar suspended in a small volume of saline. Bacterial extracts were prepared in phosphate buffer pH 7.4, 24 hr at 37° C, and the supernatants after centrifugation were used.

TABLE 1
Wall Teichoic Acids Examined Serologically

Strain	Polyol	Hydrolysis in alkali*	Sugar*
Staph epidermidis			
T1	Glycerol	Polyglycerophosphate stable to alkali	Alpha glucose
T2	"	Polyglycerophosphate stable to alkali	Beta glucose
12	"	Diglycerol monophosphate and beta glucosyl glycerol	"
7291	"	Diglycerol monophosphate and beta glucosyl glycerol	"
11249 (hyicus)	"	Glycerol diphosphate and saccharinic acid	Alpha glucosamine
Micrococcus			
13	"	Glycerol diphosphate and saccharinic acid	"
234	Ribitol	Polyribitol phosphate stable to alkali	Glucose and glucosamine

* See (2)

Antisera were prepared by injections of formalin killed bacteria in rabbits (14). Ring test precipitation and precipitin absorption were performed as earlier described (7). Agar gel precipitation in Petri dishes was performed essentially as described in (8). However when the wells were filled simultaneously with antiserum and teichoic acid in varying concentrations no precipitation lines were observed. In a series of experiments the time of application of antiserum and teichoic acid as well as the incubation temperature were then varied. The lack of precipitation lines using the standard technique was eventually found to be due to the unusually rapid migration in the agar gel of these teichoic acids. When the serum well was filled and then incubated at 3[illegible] C 3 hr prior to the filling of the antigen wells distinct precipitation lines were produced after [illegible]48 hr at 4° C even in high dilutions of the preparations.

Standard solutions of the teichoic acids and polysaccharides containing 1 mg dried material per ml were diluted in saline. T[illegible]en in dilutions of the standards

RESULTS

Staph epidermidis T1. The wall of this strain contains glycerol teichoic acid with alpha linked glucose [illegible]. Four preparations were examined: one was produced by the standard technique (1), the second by a single trichloroacetic acid extraction for 46 hr at 4° C, the third by saline extraction for 18 hr at [illegible] C and the fourth by saline extraction first for 18 hr at [illegible] C and then for 23 hr at 37° C. The saline extracts contained a trace of mucopeptide in addition to glycerol teichoic acid.

TABLE 2
Ring Test Precipitation

Teichoic acid	Serum					
	T1	T2	I2	11249	I3	Wood 46
Staph. epidermidis						
T1	6 400	–	–		–	
T2		6 400	3 200			
I2	–	6 400	6 400		–	
7291	–	3 200				
11249	–	10	–	12 800	–	1
Micrococcus						
I3	–		–		100	
234	–	400	–		–	1 600

Conc. of teichoic acids 1 mg/ml
Reciprocal titre values

TABLE 3
Agar Precipitation Lines Produced by Teichoic Acids

Line teichoic acid T1	Line teichoic acid T2	Line teichoic acid I3	Line poly A
Staph. epid. T1	*Staph. epid.* T2	*Microc.* I3	*Microc.* 234
„ 1254	„ I2		
	„ 7291		

Against antiserum T1 all four preparations produced a distinct precipitation line on agar diffusion in dilutions 1/1,600–1/3,200 and ring test precipitation in dilutions 1/3,200–1/6,400 (Tables 2 and 3). Identical results were obtained with antiserum 1254, and polysaccharide 1254 (B) gave a reaction of identity with the T1 line. None of the other teichoic acids or polysaccharides produced this line or gave a positive ring test precipitation in T1 antiserum.

T1 bacteria produced two distinct lines on agar gel diffusion against antiserum T1: one strong line on the antigen well side and a weaker one on the serum side. The latter was identical with the teichoic acid line. The strong line was produced also by 1254 bacteria but not by the other strains examined. The two saline extracts contained small amounts of the antigen corresponding to the strong line, whereas the other T1 or 1254 preparations did not produce this line. The antigen producing the strong line resisted trypsin treatment.

Cross-absorptions of T1 and 1254 antisera with the corresponding preparations resulted in negative ring tests and disappearance of the agar precipitation line, thus verifying the identity of their teichoic acid antigens. The absorptions did not remove antibodies against the strong line.

Staph epidermidis T2 The wall of this strain contains glycerol teichoic acid with beta linked glucose in the form of disaccharide residues (2) Three preparations were examined two of which were extracted with trichloroacetic acid like the T1 preparations The third preparation was obtained by saline extraction for 23 hr at 37° C

Against T2 antiserum the T2 teichoic acid preparations gave a single precipitation line on agar diffusion in dilutions 1 1 600 1 3 200 and a ring test titre of 1 6 400 The serologic activity of the 46 hr trichloroacetic acid extract was weaker in both tests The T2 teichoic acid line is identical with the precipitation line produced by teichoic acids from *Staph epidermidis* I2 and 7291, and the teichoic acid from *Micrococcus* 2H gave ring test precipitation with T2 antiserum (see below) There was no cross reaction with T1 teichoic acid

T2 bacteria gave two distinct precipitation lines on agar diffusion with homologous serum one strong line on the antigen well side and a weaker one on the serum side The latter was identical with the teichoic acid line *The strong line was not produced by the T2 teichoic acid* preparations or by the bacteria of the other strains examined *The antigen* producing the strong line was sensitive to trypsin

Staph epidermidis I2 The wall of this strain contains glycerol teichoic acid with beta linked glucose (2) A single precipitation line was produced with I2 antiserum in 1 1 600 dilution of the preparation whereas the ring test titre was 1 6 400 The line was identical with those produced by teichoic acids from *Staph epidermidis* T2 and 7291 T2 and I2 teichoic acids cross reacted to the same titres on agar gel and ring test precipitation and cross absorbed the antisera As I2 antiserum gave a prozone the T2 antiserum was preferred

I2 bacteria gave two precipitation lines on agar diffusion one strong often double line on the antigen well side and a weaker line on the serum side The latter was identical with the I2 teichoic acid line The strong line was not produced by the I2 teichoic acid preparation or by bacteria of the other strains examined The antigen producing the strong line was resistant to trypsin

Staph epidermidis 7291 Th[illegible] wall of this strain contains glycerol teichoic acid with beta linked gl[illegible] in th[illegible] form of monosaccharide residues The preparation pr[illegible] a line with T2 antiserum which gave a reaction of identity with th[illegible] T[illegible] teich[illegible] acid line and was positive in 1 3 200 dilution in th[illegible] ring test [illegible] acid 7291 completely absorbed antib[illegible] responsible for the [illegible] teichoic acid line and the ring test precipitation

Staph epiderm[illegible] 11249 (l ycus [illegible] wall of this strain contains an atypical gl[illegible] amine glycerol teichoic acid In contrast to teichoic acids from oth[illegible] stra[illegible] of *Staph [illegible] epidermidis* (2) this polymer is a mixed diester [illegible] and glucosamine phosphate (Table 1) The 11249 [illegible] r[illegible] d in high dilution with 11249 antiserum by ring t[illegible] whereas no precipitation line wa[illegible]

observed on agar diffusion. Very weak ring test reactions were observed with antisera T2, 1254, and Wood 46, whereas the ring test was negative with 13 antiserum.

Micrococcus 13. The wall of *Micrococcus* 13 contains a similar glucosamine glycerol teichoic acid as *Staph. epidermidis* 11249 (1). The serologic activity of the 13 teichoic acid/13 antiserum system was rather weak. Ring test and agar gel precipitation were positive only in 1/100 dilution of the preparation. A specific precipitation line not present in any of the other preparations was produced.

13 bacteria gave two precipitation lines on agar diffusion, one strong line on the antigen well side and a weaker one on the serum side. The latter was identical with the 13 teichoic acid line. The strong line was not produced by the 13 teichoic acid preparation or by bacteria of the other strains examined. The antigen producing the strong line was sensitive to trypsin.

Micrococcus 234. The wall of this strain contains a ribitol teichoic acid with glucosyl and glucosaminyl residues. The 234 teichoic acid gave a positive ring test precipitation in 1/1 600 dilution with Wood 46 antiserum. The polysaccharide A titre was 1/2 000. The 234 teichoic acid produced a polysaccharide A line on agar gel precipitation in 1/400 dilution. The preparation also gave a positive ring test in 1/400 dilution with antiserum T2, whereas with this serum no precipitation line was observed on agar gel. Absorption of antiserum T2 with polysaccharide A did not significantly reduce the ring test titre against teichoic acid 234.

DISCUSSION

Four of the teichoic acids here examined are of the glycerol type with glucosyl residues. In one (T1) glucose is alpha linked, in the other three (T2, 12, 7291) it is beta linked. The results indicate that in these *Staph. epidermidis* teichoic acids the serologic specificity is determined by the glycosidic linkage. There was no cross precipitation between the teichoic acids with alpha- and beta-linked glucose in the ring test and each of them produced a specific line on agar gel precipitation. The serologically specific polysaccharide 1254 was described by *Losnegard & Oeding* (11) as a glucosyl glycerol teichoic acid probably identical with *Julianelle & Wieghard*'s (10) group carbohydrate (B) present in apathogenic staphylococci. Polysaccharide 1254 was shown to react identically with teichoic acid T1 serologically, and the dextrorotation of Julianelle & Wieghard's preparation indicates that also in their preparation glucose was alpha linked. The *Staph. epidermidis* teichoic acid described by *Morse* (12) was also shown to have alpha linked glucose as the serologically determinant group.

The teichoic acids from T2, 12 and 7291, all with beta linked glucose, cross reacted completely in the ring test and agar diffusion test. In the T2 teichoic acid glucose is in the form of disaccharide residues, whereas

in strain 7291 it is in the form of monosaccharide residues. This difference apparently had no influence on the serologic specificity.

It has earlier been demonstrated that in the ribitol teichoic acid characteristic of *Staph. aureus* glucosamine is either alpha- or beta-linked and each linkage is serologically specific (13, 3). *Staph. aureus* strains appear as a rule to have both types of linkages, but certain strains have only one of these. The present investigation shows that also in the glycerol teichoic acids of *Staph. epidermidis* the sugar occurs in serologically specific alpha- and beta-linkages. The linkage may indicate two distinct types in *Staph. epidermidis* since both linkages do not appear to occur simultaneously in the same strain as demonstrated in *Staph. aureus*. It remains to be evaluated, however, whether all strains of *Staph. epidermidis* have the same teichoic acid, i.e. glucosyl-glycerol teichoic acid, or whether other types of teichoic acids are found (see strain 11249).

Micrococcus 13 and *Staph. epidermidis* 11249 (lysicus) have similar very unusual glucosamine glycerol teichoic acids. Serologically, however, they do not cross react. Glucosaminyl linkages may be the reason for the very weak precipitation of teichoic acid 11249 in T2, 1254 and Wood 46 antisera. The teichoic acids of strains 11249 and 13, in spite of their structural similarity, are completely different serologically; this must reflect a difference in their configuration resulting in different determinant groups.

The teichoic acid from the meat *Micrococcus* strain 234 cross reacts strongly in sera Wood 46 and T2. The production of a polysaccharide A line with serum Wood 46 indicates that glucosamine in teichoic acid 234 is beta-linked to ribitol and responsible for the cross reaction. Although teichoic acid 234 did not seem to produce the T2 teichoic acid line on agar diffusion, the strong ring test precipitation in serum T2 indicates that teichoic acid 234 has a second serologic specificity, probably determined by beta-linked glucose. As polysaccharide A or 263 antibodies were not demonstrated in serum T2, glucosamine is apparently not responsible for the precipitation of teichoic acid 234 in T2 antiserum.

This *Micrococcus* teichoic acid thus appears to have two serologic specificities and is related to *Staph. aureus* as well as *Staph. epidermidis* teichoic acids. A similar substance called polysaccharide AC was described by *[illegible]snegard & O[illegible]* (11). The strain from which the polysaccharide w[illegible] [illegible] to *Staph.* [illegible]
but according t[illegible]
types of te[illegible]
specificit[illegible] further investigations.

The pres[illegible] [illegible]stigation also r[illegible]ed precipitinogens present in whole bact[illegible] [illegible] with the teichoic acid antigens. The importance [illegible] for taxonomy and serologic typing remains to be evaluated. Th[illegible] demonstration of one specific precipitinogen

in the bacteria of each of the strains T2 and I2, which have a common teichoic acid antigen, indicates that the bacterial precipitinogens have a narrower specificity than the teichoic acids

SUMMARY

In glycerol teichoic acids isolated from strains of *Staph epidermidis* alpha-linked glucose had one serologic specificity, beta-linked glucose another The teichoic acids reacted in high dilutions and there was no cross-precipitation between preparations containing different linkages They produced specific precipitation lines on agar diffusion

Two very similar glucosamine-glycerol teichoic acids, one from a *Staph epidermidis* strain (hyicus) and the other from a *Micrococcus* strain were completely different serologically A ribitol teichoic acid with glucose and glucosamine from a meat *Micrococcus* apparently had two serologic specificities, one determined by beta-glucosamine linkages the other by beta glucose linkages

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THE SO-CALLED TOXOPLASMA HOSTILE FACTOR AND ITS RELATION TO ANTIBODY

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Received 30.viii.66

The effect on Toxoplasma gondii of specific antibody as revealed by the dye test is elicited only in presence of the so-called activator factors of "normal" (non-immune) serum. In the absence of antibodies the activator factors show no antitoxoplasma activity. However, *Sabin & Feldman* (21) in their first paper describing the use of the dye test for the diagnosis of toxoplasmosis also reported the occurrence of a heat-labile antitoxoplasma factor in normal sera of different mammalians. This factor was considered different from specific antibody as the antitoxoplasma activity of heat-treated normal sera could not like that of heat-treated immune sera be restored by the addition of activator serum.

Jettmar (12, 13) has described a heat-labile antitoxoplasma factor occurring in the sera of several species including man. This factor was called the toxoplasma hostile factor (THF). Jettmar in contrast to Sabin and Feldman tested the sera without the addition of activator factors. The heat lability of the THF might therefore have been due to destruction of activator factors such as heat-labile complement components originally present in the sera and necessary for the action of THF. *Gronroos* (7) reinvestigating the THF considered the antitoxoplasma activity of normal serum to be the results of the combined action of activator factors and thermostable toxoplasma antibodies both present in the sera.

The nature of the thermolabile antitoxoplasma factors described by Sabin and Feldman has not been properly established. In a review by *Feldman* (4) it was pointed out that all the sera of the animal species studied except those of mouse and man kill toxoplasma parasites in the absence of specific antibody. *Jacobs* (11) considered it possible that the antitoxoplasma activity in normal sera could be ascribed to low levels of non-specific antibody, perhaps identical with properdin.

This study was supported by a PHS research grant AI 05071-05 from the *National Institute of Allergy and Infectious Diseases, Public Health Service*, USA. The excellent technical assistance of *Miss Monica Eriksson* is gratefully acknowledged.

With respect to the information available about the heat-lability of so-called "natural" antibodies (15), reaginic antibodies (3, 23) and the relatively greater sensitivity of 19S antibodies to heat compared to 7S antibodies (20) it seemed worthwhile to reinvestigate the nature of the antitoxoplasma activity of sera from normal (non-immunized) animals

MATERIAL AND METHODS

Normal sera (Non-immune sera) Rabbit, guinea pig monkey (cynomolgus) and calf sera were obtained from apparently healthy individuals These sera were designated as normal and the factor of the sera which exerted antitoxoplasma activity, in the presence of activator factors was referred to as the toxoplasma hostile factor (THF) The dye test titres of the normal animal sera varied from 4 to 160 The highest titres were observed in the rabbit sera and the lowest in the guinea pig sera

Activator sera Human sera were obtained from apparently healthy blood donors without any previous known experience of toxoplasmosis and were tested for antitoxoplasma activity Selected sera which were free of antitoxoplasma activity were used as activator sera

Immune sera A rabbit which had no detectable serum antitoxoplasma activity in a serum dilution of 1 4 was inoculated with about 1000 living toxoplasma parasites in the marginal ear vein Ten days after the inoculation the rabbit was bled and the serum was used as a reference immune serum The serum had a dye test titre of 2000 and a titre of 128 in the complement fixation test

A human immune serum was obtained from a 24 year old man who had been diseased with the lymphoglandular form of toxoplasmosis for at least 3 months This serum had a dye test titre of 8000 and a titre of 256 in the complement fixation test

Parasites Toxoplasma parasites of the RH strain were used A toxoplasma rich peritoneal exudate was obtained by inoculating Swiss albino mice intraperitoneally with toxoplasma parasites and then after 3 days harvesting the exudate The exudate was centrifuged at 65×g to remove most of the cells and cellular debris The supernatant was recentrifuged at 440×g and the sedimented parasites were resuspended to a concentration of 5 million to 10 million parasites per ml This suspension was used in the tests described below

Zymosan A preparation type A (batch 134 84) from Koch-Light Laboratories Colnbrook England was used Absorptions of sera to get RP or R3 were accomplished using 1 ml of a 0 2 per cent suspension of boiled zymosan for each 1 ml of the serum to be absorbed

Properdin The same preparation of properdin (RdTI) (kindly supplied by AB KABI Stockholm Sweden) as described in an earlier paper (25) was used This preparation containing 16 000 units properdin per gram was devoid of demonstrable complement components as measured with the aid of conventional R reagents

Test on morphological alterations (MA) of toxoplasma parasites Antitoxoplasma activity in normal as well as in immune sera was measured by means of MA tests The test was performed as previously described (25) by mixing one part of test serum dilution with two parts of activator serum and one part of parasite suspension Determination of the percentage of parasites with MA was made after one hour's incubation at 37° C The MA observed were identical with those described as being characteristic of a positive dye test (21) The latter term is not used in the following however since no dye was used in the tests

The amount of antibody or THF giving MA in 50 per cent of the exposed toxoplasma parasites in the presence of activator serum is in the following referred to as one antibody unit and one THF unit respectively

Heat treatment An ultrathermostate LAUDA type WB 20 was used For each test 0 5 ml of the serum dilution was incubated in a glass tube for 30 minutes Dilutions of sera were performed either with Hanks balanced salt solution pH 7 0 or with activator serum The incubation temperatures employed ranged from 45° to 75° C

Reduction with 2 mercaptoethanol The method of Schrohenloher et al (22) was principally followed The serum or serum dilution was dialyzed for 3 hours at room

temperature against 0.2 M 2-mercaptoethanol in 0.15 M phosphate buffer pH 7.35. To prevent reassociation of dissociated macroglobulins, alkylation was performed by dialyzing the sera against 0.02 M iodacetamide in the same buffer for 4 hours at room temperature. Finally the sera were dialyzed overnight in the cold against the phosphate buffer used. The positive control sera were dialyzed against the phosphate buffer for 7 hours at room temperature and then overnight in the cold.

Density gradient ultracentrifugation. The separation of antibodies was performed in a Spinco model L ultracentrifuge using a preformed sucrose gradient ranging from 10 to 40 per cent. The sucrose gradient was made as described by *Britten & Roberts* (2). After addition of sucrose to a final concentration of 10 per cent the centrifugation was run for 18 hours at 35,000 rpm using a SW 39 rotor. Serial fractions, usually 13, were collected through a hole in the bottom of the tube. The fractions were tested for antitoxoplasma activity at varying dilutions.

Sephadex G-200 chromatography. Gel filtration on Sephadex G-200 was performed according to *Flodin & Killander* (6). A column of the gel 100 cm in length and 3 cm in diameter was used. In each experiment two to 10 ml of serum was applied to the column and elution was carried out in the cold with 0.15 M phosphate buffer pH 7.35. The flow rate was 3.5 ml per hour and the eluate was collected in 5 ml fractions. The protein concentration of the fractions was determined by their absorption at 280 mμ. Concentration of fractions or pooled fractions was performed either by ultrafiltration or by dialysis against 30–50 per cent polyethyleneglycol followed by dialysis against the buffer used for elution. Antibodies eluted in the first protein peak evident in the chromatogram will be referred to as 19S antibodies, and those eluted in the second peak as 7S antibodies.

Block electrophoresis. Electrophoresis of sera was performed in a Pevikon block as described by *Müller-Eberhard* (17). A 0.1 molar barbital buffer pH 8.6 was used and a potential of 5 V per cm was applied for 20–24 hours. After the run the block was cut into 1 cm wide pieces and each piece was mixed with 0.5 ml of the buffer used. After thorough shaking of the mixtures they were centrifuged and the supernatants tested for antitoxoplasma activity. The protein content of the eluates was after dilution with distilled water determined by absorption at 280 mμ. The presence of immunoglobulins in the eluates was determined by means of immunoelectrophoresis (26) with the aid of a goat anti-rabbit globulin serum obtained from Microbiological Associates, Bethesda, Md., USA.

RESULTS

Dependence of THF on Activator Factors

All the investigated undiluted normal sera had a demonstrable antitoxoplasma effect but this effect could be abolished by diluting the sera 1:2 or 1:4. The antitoxoplasma activity of the diluted sera could however be restored after addition of human activator serum.

Portions of an activator serum were treated to destroy selectively various complement components (14). The variously treated activator portions were thereafter mixed with normal rabbit serum diluted 1:2 and the mixtures were tested for their ability to cause MA of toxoplasma parasites. A completely abolished or a markedly reduced THF-activating capacity of the activator serum was encountered after treatment of the activator serum with heat (56°, 30 minutes) or NH_4OH or absorption with zymosan at 37° C (Table 1). Addition of EDTA to a final concentration of 0.02 M to a mixture of THF and untreated activator likewise resulted in a loss of the antitoxoplasma activity. A mixture of THF and zymosan absorbed (17° C and 37° C) activator regained partly its antitoxoplasma activity after addition of properdin at a final concentration of 10 units/ml.

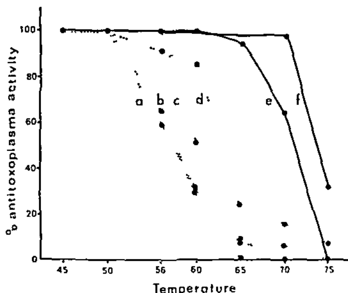


Fig 1

The sensitivity to heat of THF compared to that of immune antibodies to toxoplasma

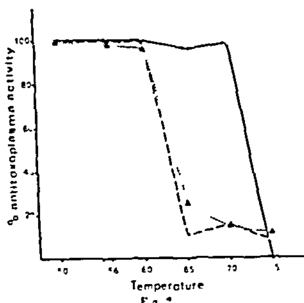
The results of heat treatment of the THF in normal calf (a) guinea pig (b) monkey (c) and rabbit serum (d) were compared with the results of treating immune antibodies in rabbit (e) and human (f) sera For each serum solutions containing four to eight antibody units were treated for 30 minutes at each temperature Antitoxoplasma activity is expressed as percentage of the activity found in the untreated serum dilution Mean values from tests on 3 normal sera from each species are plotted

TABLE 1

The Effect of the Toxoplasma Hostile Factor in Presence or Absence of Activator Factors

Sample	Per cent parasites with MA
THF	17
Activator	1
THF + activator	82
THF + activator heated at 56° C for ½ hour	10
THF + activator absorbed with zymosan (37° C)	51
THF + activator absorbed with zymosan (17° C + 37° C)	29
THF + activator treated with NH_4OH	27
THF + activator + EDTA	18
THF + properdin + activator absorbed with zymosan (17° C + 37° C)	46
Properdin + activator	6

The samples were mixed with toxoplasma parasites and after 1 hours incubation at 37° C the percentage of parasites with morphological alterations (MA) was determined for each sample Absorptions with zymosan were performed for one hour at 17° C and for half an hour at 37° C. Treatment with NH_4OH was performed according to the procedure described for obtaining R 4 (14) The final concentration of EDTA was 0.02 M and that of properdin was 10 units per ml THF stands for toxoplasma hostile factor the source of which in this experiment was normal rabbit serum diluted 1 2



F g 4

The sens t e ty to heat of THF c mpare I to those of immune 7S an I 19S ant bod es to t xoplasma.

The results cf heating n rmal ral I it serum (d tted line) and immune 19S br ken and 7S sol d line ant bod es are c mpare I S luti ns c ntaining f ur ant body un ts were treated f r each serum cr serum fracti n at each temperature f r 30 m nutes The 7S and 19S ant bod es were c ntained in fracti ns f Sephadex G 200 chr mat graphy of rabl it immune serum Antitox plasma activity is expresse I as percentage f the activity f und in the untreated serum I luti n r serum fracti n

Abs rption of THF with Toxoplasma Parasites

Normal unheated fresh ral bit sera in varying dilutions were absorbed with a high concentration of toxoplasma parasites (× 10 parasites per ml) for 1 hour at 37° C. Absorption of sera containing up to two THF units with equal volumes of parasite suspension resulted in a total removal of the THF activity The THF could be absorbed also when the absorption was performed at 4

Influence of Heat on THF

Normal animal sera 3 each of calf guinea pig monkey and rabbit origin were heat treated at the temperatures indicated in Fig 1 for one-half hour The sera had been diluted in Hanks solution or human activator serum to contain 2–4 THF units For comparison two immune sera containing both 7S and 19S antibodies as judged by Sephadex G 200 chromatography were also tested The reults of testing the anti toxoplasma activity of the treated sera in the presence of activator serum are shown in Fig 1

It is evident from the figure that the THF in sera from the various animals showed different degrees of heat lability Compared to the

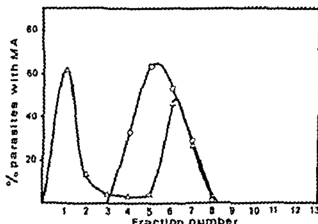


Fig 3

Density gradient ultracentrifugation of THF and immune antibodies to toxoplasma

The results of ultracentrifugation in a sucrose gradient of one immune rabbit serum and one normal rabbit serum are illustrated. The antitoxoplasma activity is indicated as *percentage of toxoplasma parasites showing MA after exposure to the* serum fractions in the presence of activator. The immune serum tested was in other experiments by gel filtration (Fig. 4b) shown to contain 7S as well as 19S toxoplasma antibodies. Circles indicate antitoxoplasma activity in normal serum fractions and *triangles* indicate activity in immune serum fractions diluted 1:20 before being tested.

antibody activity in the immune sera tested, the THF activity in the normal sera was in all cases considerably more sensitive to heat.

In order to further characterize the heat-sensitivity of the THF in relation to those of 7S and 19S antibodies, Sephadex G 200 fractions of the immune rabbit serum containing these antibodies and a normal rabbit serum were heat treated. Before heating the fractions were diluted to contain equal amounts of antitoxoplasma activity. As shown by Fig. 2 the 19S antibodies were more sensitive to heat than the 7S antibodies. The THF activity of normal rabbit serum had a sensitivity to heat similar to that of the 19S immune antibody activity.

Reduction with 2-Mercaptoethanol

The THF in all the normal rabbit, guinea pig, calf and monkey sera studied was found to lose its activity after treatment with 2-mercaptoethanol. Experiments with 7S and 19S Sephadex fractions of the rabbit immune serum showed that the 19S antibody activity was completely abolished by treatment with 2-mercaptoethanol. At the same titre levels the 7S antibody titres were, however, also affected by this treatment and diminished about four- to eight-fold.

Density Gradient Ultracentrifugation

Two normal rabbit, one guinea pig, one monkey and one calf serum as well as the immune sera of human and rabbit origin were fraction-

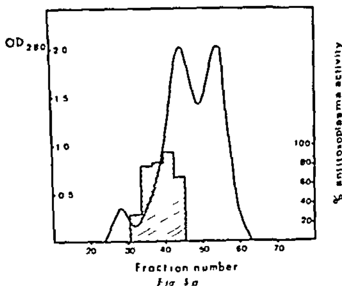


Fig 4 a

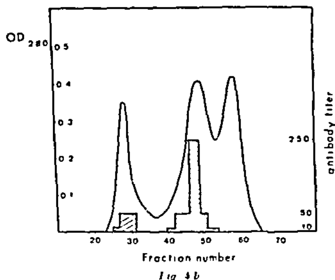


Fig 4 b

Fig 4

Gel filtration of THF and immune antibodies to toxoplasma

The results of chromatography on Sephadex G 200 column of normal rabbit serum (4 a) and rabbit immune serum to toxoplasma (4 b) are illustrated. Solid curve represent optical density at 280 mμ. The antitoxoplasma activity measured as relative activity of undiluted fractions (Fig 4 a) or MA test titre of the fractions (Fig 4 b) is illustrated by hatched areas.

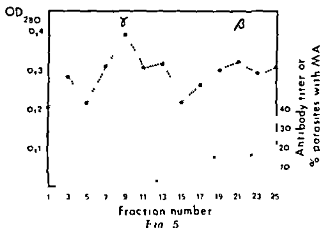


Fig 5

Pevikon block electrophoresis of THF and immune antibodies to toxoplasma

The electrophoretic mobilities of antibodies in rabbit immune serum and the THF in a normal rabbit serum are compared

The antitoxoplasma activity is expressed as MA test titre for the fractions of the immune serum or for the normal serum fractions, as percentage of toxoplasma parasites showing MA after exposure to the fractions in the presence of activator Hatched area surrounded by dotted line indicates immune antibody activity and hatched area surrounded by solid line indicates THF activity Dotted curve represents optical density at 280 mμ of diluted fractions

ated by ultracentrifugation in a sucrose gradient The THF or antibody activities of the fractions obtained were determined The THF in all sera tested had a sedimentation rate which was equal to or slightly faster than that of the 7S antibodies Fig 3 shows the results of an experiment where the sedimentation of the THF in normal rabbit serum is compared with the sedimentation of the 7S and 19S antibodies of immune rabbit serum

Sephadex G 200 Chromatography

Normal sera from 5 rabbits one monkey, one calf and 2 guinea pigs were fractionated by gel filtration on a Sephadex G 200 column The antitoxoplasma activity was in all cases, except for one rabbit serum, which showed activity in both the first and second peak, located in fractions eluted slightly ahead of the second peak and extending into this peak The maximum activity was for all the four rabbit sera which had only one peak of activity, eluted ahead of the second peak Representative results are shown in Fig 4 which illustrates the results of gel filtration of one normal and one immune rabbit serum

Block Electrophoresis

Normal sera from 2 rabbits, one monkey, one calf and one guinea pig as well as rabbit immune serum containing both 7S antibodies and 19S antibodies, were subjected to Pevikon block electrophoresis By this technique the THF in the normal rabbit sera studied was found to have

a mobility similar to β-globulin see Fig. 5. The zone of maximum activity was distinctly different from the zone of maximum activity of the immune serum tested. The mobilities of the THF in the monkey calf and guinea pig sera were similar to γ-globulins.

DISCUSSION

The present investigation has given results indicating that what in this study has been called the toxoplasma hostile factor (THF) is a serum globulin which can be adsorbed to toxoplasma parasites and is dependent on complement factors for its action. The factor seemed in almost all the sera investigated to have characteristics which were different from those of IgM (19S antibodies). The results concerning the THF in rabbit sera are consistent with the hypothesis that the THF may be a fast moving immunoglobulin possibly of the IgA type. No conclusions regarding the frequency of the occurrence of THF being this type of immunoglobulin in various animals can be drawn. It should be noted however that of all normal animals tested only one had serum THF activity in IgM fractions. Since the factor occurs in normal serum it may tentatively be discussed as "natural" antibody although it cannot be excluded that the animals studied had previously suffered from toxoplasma infection. Natural antibodies have in recent studies (16, 24) been shown to be immunoglobulins mainly of the IgM variety. Rabbit antibodies of the IgA type have been demonstrated by others. *Onoue et al.* (18) found that rabbit antihapten antibody could be IgA with a sedimentation coefficient of either 7S or 9S. *Zvaifler & Becker* (28) reported results suggesting that rabbit antibodies capable of sensitizing rabbit skin for passive cutaneous anaphylaxis may be IgA.

Natural antibodies have in earlier studies been reported to be heat labile. *Michael et al.* (15) stated that "for each species immune antibody is more heat stable than natural antibody". They found a considerable variation between different species regarding the heat stability of natural antibodies. These results fit well with those of the present investigation where the toxoplasma hostile factors from different species showed different degrees of heat lability but were always more heat labile than the immune 7S antibodies tested. The differences noted could not be ascribed to higher concentrations of antibody in the immune sera than in normal sera since all sera were tested in dilutions containing approximately the same number of antibody units.

The heat lability of the natural antibodies against toxoplasma should be discussed in relation to the effect of heat on other antibodies studied. *Pike & Schulze* (20) found that 19S antibodies against various antigens were more heat labile than 7S antibodies against the same antigens. *Svehag* (24) reported that natural antibodies against poliovirus in rabbit sera were 19S globulins and were more heat labile than immune antibodies. Reaginic antibodies are markedly heat labile and are usuall

sensitive to heating at 56° C for 30 min. (3, 23) There is suggestive evidence at hand that human reaginic antibodies usually are IgA (5, 8), although the recent findings reported by *Ishizaka & Ishizaka* (10) indicate that they may be immunoglobulins of another type

The apparent sensitivity of toxoplasma natural antibodies to treatment with 2-mercaptoethanol might indicate that they are IgM or IgA However, as *Wiederman et al* (27) have pointed out, the results of this sort of treatment do not offer any conclusive evidence regarding the nature of complement-fixing antibodies as it may very well occur that the complement-fixing capacity of IgG antibodies is destroyed by 2-mercaptoethanol without any effect on their combining efficiency It was found in the present study that in most cases there was an evident lowering of the 7S antibody titres after treatment with 2-mercaptoethanol

The THF in rabbit sera seemed to be dependent upon complement *for its action although there was experimental evidence that it was neither IgG nor IgM This finding is important in relation to the fact* that in previous investigations (1, 9) IgA antibodies have not been found to fix complement The rabbit skin-sensitizing antibodies described by *Zvaifler & Becker* (28) did not fix complement

Concerning the origin of the THF nothing is known As regards natural antibodies to viral antigens *Svehag* (24) has proposed that these antibodies are "associated with an immunological response to repeated stimulation with minute amounts of cross-reacting or identical antigenic determinants" The THF may possibly have a similar origin The present results raise the question whether low serum dye test titres represent results of toxoplasma infection or merely stimulation by toxoplasma antigens present in foods Low serum dye test titres are undoubtedly in many cases the result of a toxoplasma infection However, the conclusions of any serologic survey using the dye test, with the aim to determine the prevalence of toxoplasma infection should be restricted to the findings of those higher titres commonly associated with generalized toxoplasma infection

Properdin has by several authors been considered as being identical with natural antibody In the present investigation properdin mixed with activator serum had no morphologically demonstrable significant effect on toxoplasma parasites in contrast to THF and activator A properdin preparation previously (25) found to be devoid of demonstrable complement components could partly restore the antitoxoplasmic effect of a mixture of natural antibodies and a zymosan-absorbed activator serum Thus it seems clear that properdin and natural antibodies to toxoplasma are different entities The claim that properdin is a distinct serum entity is in accord with the results of *Pensky et al* (19) who found that properdin does not share antigenic determinants with the known immunoglobulins

SUMMARY

The so-called toxoplasma hostile factor was investigated in rabbit guinea pig calf and monkey sera The factor was found to be antibody to toxoplasma showing different degrees of heat lability in various sera By means of gel filtration block electrophoresis density gradient centrifugation and reduction with 2 mercaptoethanol the factor was in almost all cases shown to have characteristics different from those of IgM antibodies The results suggested that the toxoplasma hostile factor in rabbit sera may be antibody of the IgA type The dependence on complement and properdin for the action of the factor was demonstrated The results indicate that the toxoplasma hostile factor and properdin are different entities The origin of the toxoplasma hostile factor is discussed

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BRIEF REPORTS

COUNTING THE NUMBER OF AEROBIC BACTERIA IN FULL-THICKNESS HUMAN EPIDERMIS SEPARATED BY SUCTION

By *K. K. Mustakallio, O. P. Salo, Raija Kiistala* and *U. Kiistala*

Both dry and fluid sampling methods have been used for the study of the number of skin bacteria. The impression techniques illustrate the density of the bacteria on the skin surface but do not differentiate between single bacteria and bacterial aggregates. The scrubbing, scraping and tape-stripping methods, while giving some idea of the deeper flora, suffer from the same drawback. In the scrubbing technique the application of a sampling fluid on the skin surface causes some dispersion of bacterial aggregates but, on account of individual variations in the penetration of the fluid, it introduces uncontrollable factors.

TABLE

Counts of Aerobic Bacteria in Full Thickness Epidermis

Number of case	Sex and age	Diagnosis	Site of suction biopsy	Area of biopsy in cm	Bacteria per cm
1	♀ 30	Healthy	Midvolar forearm	0.18	216
2	♂ 21	Healthy	Midvolar forearm	0.35	940
3	♂ 27	Healthy	Midvolar forearm	0.36	83
4	♂ 23	Healthy	Midvolar forearm	0.41	1370
5	♂ 33	Healthy	Midvolar forearm	0.41	146
6	♂ 25	Healthy	Midvolar forearm	0.59	119
7	♂ 32	Healthy	Midvolar forearm	0.4	64
8	♀ 18	Healthy	Midvolar forearm	0.40	5
9	♂ 23	Healthy	Midvolar forearm	0.45	194
10	♂ 27	Healthy	Midvolar forearm	0.47	83
11	♂ 15	Healthy	Midvolar forearm	0.31	37
12	♀ 69	SLE	Upper abdomen	0.15	67
13	♀ 76	Leg ulcer	Upper abdomen	0.65	3100
14	♂ 69	Pruritus	Midvolar forearm, left	0.45	860
			Midvolar, right	1.00	700
15	♀ 14	Atopic dermatitis	Midvolar, left, site a	0.80	5100†
			Midvolar, left, site b	0.80	3400†
			Midvolar, right	0.75	2900†
16	♂ 12	Atopic derm.	Upper abdomen	0.04	250*
17	♂ 18	Atopic derm.	Midvolar forearm§	0.04	3 250†
18	♀ 16	Atopic derm.	Midvolar forearm§	0.07	1 0000*

— Skin of normal appearance
§ — Lichenified skin
† — *Staphylococcus aureus* present among other bacteria
* — Pure culture of *Staphylococcus aureus*

Received 30.1.67. From the *Department of Dermatology, University Central Hospital*, Snellmaninkatu 14, Helsinki 17, and from the *Department of Hygiene, University of Helsinki*, Helsinki, Finland.

[illegible]

kept in a non aggregated but viable condition

This note describes a suction biopsy technique fulfilling these requirements Suction measuring 150-200 mmHg below atmospheric pressure results within three hours in a clean dermo-epidermal separation (1) The roof of the suction blister consists of a non-contaminated full thickness sheet of epidermis The blister base reveals the original area of the epidermal sample To recover the bacteria in a dispersed state the sample is gently homogenized in a glass homogenizer with a known amount of 0 075 M phosphate buffer (pH 7 9) containing 0 1 per cent of a non-ionic detergent Triton X 100 This solution has been successfully used for the dispersion of bacterial aggregates in a recent modification of the fluid scrubbing method (2) According to our experience it does not either alter the viability of aerobic bacteria within 24 hours In the presence of the detergent the recovery of the bacteria was found to be about threefold as compared with counts obtained with buffer solution only Homogenization was found to have no effect on the recovery of bacteria from the detergent solution

The variation of the numbers of aerobic bacteria in the full thickness epidermal biopsies is illustrated in the table

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Acta path et microbiol scandinav *69* 478 479 1967

IN OVO INHIBITION OF THE GROWTH OF TRIC AGENTS BY PURIFIED SPECIES SPECIFIC INTERFERON

By V *Reinicke C H Mordhorst* and *F Schonne*

TRIC (*Trachoma Inclusion Conjunctivities*) agents constitute a group of organisms which are not true viruses but agents which have certain developmental and biochemical criteria in common with rickettsia and bacteria.

Previous experiments in this laboratory failed to show sensitivity of TRIC agents grown in ovo to relatively small virus inhibiting doses of crude chick interferon (2) Recently however sensitivity to the effect of interferon was demonstrated for a tissue culture (L-cell) grown TRIC-agent (I B 1) in experiments employing high titred mouse interferon (1) Reinvestigation of the problem of in ovo sensitivity of TRIC agents has been performed in this laboratory using purified concentrated chick interferon (3) Crude interferon was prepared in chick embryos inoculated with influenza B Lee virus and purification was carried out by a six stage procedure consisting of 1 precipitation of virus and inactive proteins with 0 15 n $HClO_4$ 2 precipitation of interferon with Zn and 3 resolution in HCl followed by dialysis to remove the Zn The Zn precipitation 4 and resolution [5] were carried out twice and the material from the second precipitation was finally lyophilised 6 and used as purified interferon The titres (50 per cent plaque inhibition end points of crude interferon and purified interferon were 1 200 (200 units of interferon per ml) and 1 63 000 (63 000 units of interferon per ml) respectively

The first experiments were concerned with the influence of interferon on the titres (ELD_{50} — Egg Lethal $Doses_{50}$ of a stock suspension of the trachoma strain

Received 24 I 67 from *the Influenza Department and the Ornithosis Department Statens Seruminstitut Copenhagen Denmark* and *the Department of Virology Rega Institute University of Louvain Louvain Belgium*

BOLB Repeated experiments with varying doses of the BOLB strain showed that 1200 units of interferon inoculated into the yolk sac of the chick embryos (either three hours before or simultaneously with the BOLB strain) resulted in a clear cut growth inhibition of the BOLB agent. A representative experiment showed a titre of $10^{-6.5}$ ELD$_{50}$ per ml in the control embryos in contrast to 10^{-2} ELD$_{50}$ per ml in the interferon treated embryos. Similarly purified interferon (1200 units) also decreased the death rate of TRIC agent SS LON inoculated embryos as revealed by a delay of two days in 50 per cent embryo mortality (after infection with 10^{-3} ELD$_{50}$) in the interferon treated group (Table 1). Experiments failed to show any direct inactivation of TRIC agents by interferon. Interferon in amounts with a definite inhibitory effect on the growth of the BOLB strain in chick embryos did not influence the growth of BOLB in duck embryos (6). Two important criteria for inhibitory effect attributable to interferon were consequently fulfilled in the experiments described: 1) Lack of direct inactivating properties and 2) species specificity of the action.

Preliminary studies of the amount of interferon necessary for in ovo inhibition of TRIC agents were also performed. It was found that > 600 units of interferon per embryo resulted in growth inhibition while < 300 units had no detectable inhibitory effect. Earlier experiments have shown that detectable inhibition of in ovo growth of influenza virus was obtained with a minimum of 50-100 units of interferon per embryo (5). Thus TRIC agents seem to be considerably less sensitive than influenza virus to the action of interferon.

With the accumulating evidence that TRIC agents are not viruses but organisms resembling bacterias (4), it is interesting that interferon, generally considered to be a virus inhibiting substance, is able to inhibit the growth of these agents. It remains a question whether the present observation can be extended to other intracellularly grown organisms that differ from viruses.

TABLE 1

The Influence of Interferon on the Death Rate of TRIC Agent SS LON Inoculated Chick Embryos

Days after inoculation of SS LON (10^{-3} ELD50/embryo)	Number and day of death of chick embryos	
	Interferon treated	Control
3		
4		
5		1
6		2
7	1	4
8	3	5
9	2	1
10	5	
11		
12	2	

The dotted lines show the time of 50 per cent embryo mortality.

The authors wish to thank Mrs. *I. Søby*, Miss *L. Christensen* and Mr. *D. Sluyts* for skilled technical assistance.

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Acta path. et microbiol. scandinav. 69, 480, 1967

HAIR NEOGENESIS IN RAT AND RABBIT SKIN

By F. Stenback, T. Nuunimäki, K. Dammert

Until recently, the consensus has been that the population of hair follicles in the adult animal is fixed, the number being determined during foetal life. However, new formation of hair follicles has been reported by some authors. Some of the most important experiments in this field are those of *Breedis* (1) and *Taylor* (3). Taylor studied the healing of the skin of rat following *freezing*, while Breedis' studies were based on healing of full thickness wounds in rabbit skin.

The present authors have repeated these experiments in order to study hair neogenesis in different conditions. The skin of 20 rats was frozen with carbon dioxide snow following the method described by *Taylor* (3). A carbon dioxide slab, diameter 1 cm, was pressed onto the back skin of the rats with constant gentle pressure for 1 minute. This caused necrosis of the skin and a wound formed. Gradually the edges contracted and after 10 weeks the wounds were almost completely healed. Histological sections of the wounds were taken weekly for 10 weeks. Neither macroscopical studies nor the histological sections showed any signs of hair formation in the granulation tissue. In contrast to the studies of *Mikhail* (2) and *Taylor* (3) *no neogenesis of rat hair could be observed.*

Using rabbits, a series of experiments was performed by the technique of *Breedis* (1). A metal ring was placed in the wound made in the back skin of 10 rabbits. The wound was protected by a glass cover. The animals were studied for 6 months. During the first days the upper layer of the loose subcutaneous tissue became attached to the loose edges of the wound. In 7–10 days the *membrane* thickened and became opaque. In 10–14 days a brown scab covered the entire wound. The granulation tissue growing in from the edges reached the centre in about 3 weeks. Epithelialization was usually complete a week later and the scab disappeared. In most animals small hairs became visible after about 2 months. They were white and no pigment could be seen. After 6 months a smooth covering of white hair covered the entire surface of the wound.

The present investigations support the concept of *Breedis* (1) that scar epithelium is capable of redifferentiating into hair follicles and sebaceous glands and that granulation tissue is capable of redifferentiating into dermal papillae. These findings indicate that cutaneous cells are pluripotential and equipotential throughout life, at least in rabbits.

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Received 13.1.67. From *Department of Pathology, University of Oulu*.

Acta path. et microbiol. Scandinav. 69: 481-488, 1967

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ROUS RAT SARCOMA STUDIED WITH FERRITIN CONJUGATED ANTIBODIES

By

Lars Göran Lindberg and Peter Biberfeld

Received 5.ix.66

Certain strains of Rous Sarcoma virus (RSV) are known to induce sarcomas in rats (for references see *Zilber* 1965). Transfer of Rous rat sarcoma cells to chickens may elicit a Rous chicken sarcoma at the site of injection indicating the presence of virus information in the rat sarcoma cells. It is, however, not known in what form the virus information persists in the rat sarcoma. So far infectious virus has not been isolated from the rat tumors. Nor have virus particles been demonstrated by the electronmicroscopic technique in the rat sarcoma cells. An attempt to demonstrate the presence of the virus using the fluorescent antibody technique was made in a previous study (*Lindberg* 1964). Antibodies against Rous sarcoma virus were produced in chickens. These antibodies were tested on living rat sarcoma cells which showed a specific fluorescence in the periphery of the cells. It was suggested that the rat sarcoma cells contained an antigen common to both chicken and rat sarcoma cells.

The aim of the present study was to investigate the occurrence of RSV antigens in the Rous rat sarcoma cells using ferritin labelled antibodies. The rat sarcoma was induced in 1961 with RSV strain S. R. by *Ahlström & Jonsson* and has since then been carried in series in rats. Transfers of the rat sarcoma to chickens almost invariably induced a virus producing Rous chicken sarcoma.

MATERIAL AND METHODS

Virus. Rous virus (RSV) strain SR was obtained from homogenates of rapidly growing chicken sarcomas purified by slow speed ultracentrifugation according to *Moloney* (1960). The virus pool was suspended in 0.0 M citrate buffer at pH 6.8 and stored at −70° C until used. Its activity after storage was more than 10⁴ FFU/ml when tested according to *Temin & Rubin* (1958).

RR sarcoma. The Rous rat sarcoma has the character of a rapidly growing

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This investigation was supported by research grant 6467 from the Swedish Cancer Society and by research grant CA 06415-04 from the National Institutes of Health, U.S.A. and grants from the Lotten Bohman Foundation.

anaplastic round cell sarcoma which usually kills the rats in 10–14 days. Retransfer of living rat sarcoma cells from every 10th passage into chicken induces a Rous chicken sarcoma of the same histological appearance as the original chicken sarcoma. The investigation was carried out on rat sarcoma cells obtained from ascitic fluid. The solid rat tumor (passage Nos. 124 and 125) was finely minced with scissors and suspended 1:5 in phosphate buffer saline. 0.1 ml of the suspension was injected intraperitoneally into rats, aged 3–4 days. Ascitic fluid containing numerous sarcoma cells, almost 100 per cent of which were viable, was obtained seven days later.

RSV antiserum. Antibodies against RSV were produced in chickens at least 10 weeks old, immunized according to the scheme suggested by *Fink & Rauscher* (1961). After three booster injections given at 3-week intervals the chickens were bled and serum was tested for virus neutralizing capacity. Serum diluted 1:100 gave at least 90 per cent inactivation. The test was performed according to *Rubin* (1960).

Antiserum against chicken γ globulin. Antibodies were produced in rabbits. The chicken globulin was purified according to *Strauss et al.* (1960). Immunization was done in a conventional way and the animals were bled when signs of an anaphylactic reaction was noticed after a small intravenous booster injection. The specificity of the rabbit serum was tested by immuno-electrophoresis and the double diffusion technique in agar gel according to *Ouchterlony* (1949). Only serum producing a single clear-cut precipitation line against chicken γ globulin was accepted for further use.

Conjugation of immunoglobulin and characterization of conjugates. The procedure used for the conjugation of antibody and ferritin and the purification of conjugates is described in detail elsewhere (*Biberfeld et al.* 1966 a, b). Briefly, the globulin fraction used for conjugation was obtained by sodium sulphate precipitation of the immune serum (*Strauss et al.* 1960). Ferritin (Pentex Inc., Kankakee) and globulin were conjugated by means of the bifunctional reagent toluene-2,4-diisocyanate (TC) essentially according to the procedure described by *Singer & Schick* (1961). Unconjugated globulin was removed by ultracentrifugation of the crude conjugate at 110 000 g for 1½ hours.

The conjugate was fractionated further by column electrophoresis.

Only fractions immunophoretically free from unconjugated ferritin and globulin respectively, were used for incubation experiments. Agar diffusion tests according to *Ouchterlony* (1949) against anti-ferritin serum and anti-rabbit γ globulin serum confirmed the purity of the conjugate and precipitin lines showing partial fusion were obtained.

Testing of sarcoma cells. The rat sarcoma cells were tested for cellular antigens using the fluorescent antibody technique as a model (*Möller* 1961). After the rat sarcoma cell suspension had been thoroughly washed in BSS (balanced salt solution) about 2×10^6 cells were incubated with chicken anti-RSV serum for 20 minutes at 37° C. The suspension was then washed three times in BSS and then incubated with ferritin labelled antibodies for another 20 minutes at 37° C, after which it was rewashed and immediately fixed and processed for electron microscopy.

In control experiments the sarcoma cells were treated with saline or chicken anti-rabbit albumin serum prior to the incubation with the ferritin conjugate. The specificity of the reaction was also tested by blocking experiments with unconjugated anti-chicken γ globulin serum.

Electron microscopy. The cell suspension was fixed in glutaraldehyde (6.5 per cent in saline) (*Sabatini et al.* 1963) for about 3 hours at 4° C and then post fixed in buffered OsO_4 (3.33 per cent OsO_4 in s-collidine, pH adjusted to 7.2 with phosphate buffer) for 60 minutes, dehydrated in graded ethanol and embedded in Epon 812 (*Luft* 1961). The specimens were sectioned on a LKB Ultrotome and examined in a Zeiss EM 9 electron microscope.

RESULTS

The rat sarcoma suspensions, incubated first with anti-RSV-antibodies and then with ferritin-labelled anti-chicken-γ-globulin antibodies, contained cells with conglomerates of ferritin, which appeared to be irregularly attached to a filamentous, branched substance (see Figs. 1, 2).



Figs 1 to 4 illustrate details of Rous rat sarcoma cells which have been treated with chicken anti RSV serum washed and subsequently inculated with purified ferritin conjugated anti-chicken antibodies

Fig 1

Rous rat sarcoma cell showing conglomerates of ferritin located in the outer third of the cytoplasm × 40 000 Detail of same conglomerates × 35 000

Practically all the conglomerates were situated in the periphery of the cell as a rule just inside the outer cell membrane and never in the central part of the cell Sometimes the ferritin particles and conglomerates were localized inside vesicles in the ectoplasm and as a rule only in those which were located close to the plasma membrane Sometimes the ferritin conglomerates adhered to the vesicle walls No tagging of the plasma organelles or of structures resembling RSV particles was observed

In some of the sarcoma cells rather large conglomerates of ferritin were found as seen in Fig 1 In these cells the cell matrix around the conglomerates seemed to be looser or less homogenous than in normal tumor cells (see Figs 1 and 2) Careful study of the cell membrane of these cells revealed small parts of the membrane which seemed to be

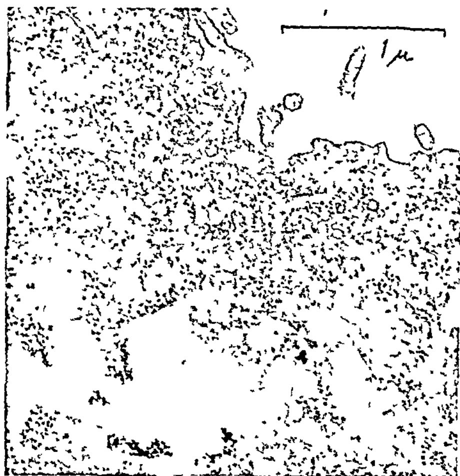


Fig. 2

Conglomerates of ferritin particles arranged like filaments in the outer part of a Rous rat sarcoma cell. No conjugation to specific cell organelles. Note the swollen and loose appearance of the cell matrix around the ferritin conglomerates. × 40 000.

split or porous (see Figs. 3 and 4). The appearance of the plasma membrane resembled that demonstrated by *Jacobsson & Wahren* (1965) in experiments with Gross lymphoma cells treated with cytotoxic tumor specific antibodies.

In cells classified as dead the ferritin particles were scattered in the cytoplasm as well as in the nucleus. These cells usually showed vacuolization and disintegration of the cytoplasmic organelles, swollen mitochondriae and parts of the plasma membrane disrupted or totally lacking. As a rule cells which were viable when fixed could readily be distinguished from dead cells. The random distribution of ferritin-conjugates in the cytoplasm of the dead cells is probably an unspecific diffusion phenomenon in analogy with the fluorescence of the nuclei and cytoplasm in dead cells in experiments using the fluorescent anti-



Figs 3 4
Part of Rous rat sarcoma cells tagged with ferritin and showing porous parts of the cell membrane × 45 000 and 60 000 respectively

body technique (*Möller* 1961) Apart from cells classified as dead there were hardly any cells in the control preparations containing appreciable amounts of ferritin

No ferritin was observed in sarcoma cells treated with saline When unspecific immune serum (chicken anti rabbit albumin) was used instead of anti RSV serum occasional ferritin particles were seen in the cytoplasm of the sarcoma cells The percentage of tagged cells however, was significantly lower and only exceptionally did they contain conglomerates comparable in size and appearance to those illustrated in Figs 1 and 2

Sarcoma cells treated with chicken anti RSV serum and subsequently incubated with unconjugated anti-chicken γ globulin serum lost all their ability to bind ferritin conjugated anti-chicken globulin Thus the ferritin-conjugated antibodies were prevented from reacting with the anti RSV antibodies in the sarcoma cells

Unlike previous fluorescent studies (*Lindberg* 1964) it was not possible to determine the number of cells reacting with anti RSV serum However the number of cells with specific ferritin staining was roughly the same as that found to be positive in the fluorescence experiments or about 20 per cent of the sarcoma cells reacted with anti RSV serum

DISCUSSION

Earlier experiments using the fluorescent antibody technique have indicated that Rous chicken sarcoma cells and Rous rat sarcoma cells contain determinants with identical immune properties (*Lindberg* 1964)

In the present study the distribution of the RSV antigens in the rat sarcoma cells were investigated by means of the ferritin antibody

technique In the rat sarcoma cell the ferritin particles were intracellularly localized to the peripheral zone of the cytoplasm and to cytoplasmic vesicles just below the cell membrane. This might indicate an antigen-antibody reaction in this region, which is in agreement with the previous immunofluorescent observations, where the fluorescence was found along the cell membrane Some of the cytoplasmic vesicles, even those containing ferritin, might represent pinocytotic vesicles and it is possible that pinocytosis could in part explain the intracellular localization of the ferritin into the cell However, mere pinocytosis cannot explain the fact that only cells treated with anti-RSV-serum contained vesicles with conglomerates of ferritin

The intracellular localization of the ferritin conjugate implies injury to the cell membrane, presumably accompanied by altered osmoregulation (*Goldberg & Green* 1960) The RR-cell incubated with anti-RSV-serum showed slight membrane changes, resembling those described by *Jacobsson & Wahren* (1965) In addition the swollen appearance of the cytoplasm might indicate osmotic damage

The results obtained so far do not allow any definite conclusions about the nature of the presumed antigen

No Rous virus particles have been found, and no strucures resembling virus particles have been seen in association with the ferritin conglomerates It cannot, however, be excluded that the intracellular antigen is related to an incomplete Rous virus particle The peripheral localization of the ferritin conglomerates indicates that the antigen is accumulated at the periphery of the cells By histochemical staining it is possible to demonstrate in the Rous rat sarcoma cell a large amount of acid mucopolysaccharides, which has almost the same distribution as the ferritin conglomerates Both the Rous chicken sarcoma and the Rous rat sarcoma are known to produce acid mucopolysaccharides, and an association between them and the antigen cannot be excluded

The intracellular localization of the ferritin conglomerates must not be taken as definite evidence of an intracellularily located antigen It is possible that the ferritin conglomerates owing to injury of the cell membrane have entered passively into the cell The loose appearance of the cytoplasm is consistent with such an interpretation It is evident, however, that a specific antigen-antibody reaction has occurred as no reactions at all could be observed in the control preparations

Recent experiences using the transplantation technique indicate the presence of cellular antigens common to Rous mouse sarcoma and Rous rat sarcoma cells (*Jonsson* 1966) indicating that the sarcoma cell has antigenic determinants on the cell membrane, which can react with specific antibodies The demonstration of specific complement-fixing antigens in hamster and guinea-pig tumors (*Huebner et al* 1964) also indicates the presence of common antigens However the specific anti-RSV-serum used in the present investigation was produced against purified homogenates of Rous chicken sarcoma tissue Moreover, no

common transplantation antigens could be demonstrated between Rous chicken and Rous mouse sarcoma (*Jonsson* 1966) The precise nature and localization of the antigen (surface antigens ?, incomplete virus ?, antigens related to or connected with mucopolysaccharides ?) is an open question

SUMMARY

The occurrence and localization of antigens in Rous rat sarcoma cells reacting with anti RSV-antibodies were studied by the ferritin antibody technique The antisera were obtained in chickens immunized with partly purified chicken sarcoma homogenate The ferritin conjugated antibodies were found to react with some of the rat sarcoma cells The reaction occurred at the periphery of the tumor cell but did not involve specific cell organelles or any structures resembling intact Rous virus particles

The results indicate the presence of antigens common to Rous rat sarcoma and Rous chicken sarcoma

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MALIGNANT CATECHOLAMINE PRODUCING TUMOUR OF THE CAROTID BODY

By

C. A. Hamberger, C. B. Hamberger, J. Wersäll and J. Wågermark

Received 2.ix.66

Tumours of the carotid body are usually benign, slowly growing neoplasms (*Le Compte* 1951). Bilateral as well as multilocular forms have been reported (*Marcuse & Chamberlain* 1956, *Rush* 1963). Malignancy is rare and only a few cases have been reported in the literature (see review by *Pryse Davies et al.* 1964).

Carotid body tumours producing catecholamines have been described earlier (*Berdal et al.* 1962, *Glenner et al.* 1962, *Pryse Davies et al.* 1964). The present paper deals with such a malignant carotid body neoplasm which was studied with light microscopy and with the fluorescence method for the demonstration of certain monoamines (*Falck et al.* 1962, *Falck* 1962, *Corrodi & Hillarp* 1963, 1964).

CASE REPORT

The patient was a 65 year-old man suffering from hypertension (210/120). Among laboratory tests may be mentioned the urinary excretion of noradrenaline which was 211 µg per day (normal 33 ± 11 µg per day). He was operated upon for a carotid body tumour growing invasively around and in the adventitial layers of the right carotid artery. At the operation the carotid bifurcation had to be resected and replaced by a vein graft. The patient died four days afterwards from extensive infarction of the brain.

MATERIAL AND METHODS

Specimens were taken for light microscopy from the tumour during the operation and from the operation field and the lymph node metastases at the autopsy. These

The investigation was supported by grants from the *Swedish Cancer Research Foundation*, the *Swedish Medical Research Council 12x 711-02* and *U.S. Public Health Service Grant NB 03956-03*.

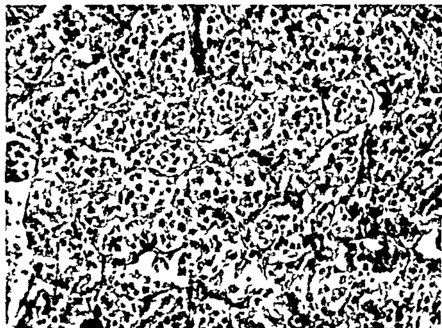


Fig. 1

Carotid body tumour composed of groups of round or polygonal epithelioid cells with a clear cytoplasm. Hematoxylin and Eosin. 200 ×

specimens were fixed in 10 per cent neutral formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

During the operation small parts of the carotid body tumour were also quickly frozen in propane cooled with liquid nitrogen, freeze-dried, treated with formaldehyde gas at 80° for one hour, embedded in paraffin wax, sectioned and mounted for fluorescence microscopy. (For further technical details see *Norberg & Hamberger* 1964, *Falck & Owman* 1965.)

Urinary catecholamine excretion was determined according to *Euler & Lishajko* (1961).

RESULTS

The carotid body tumour was composed of groups of round or polygonal epithelioid cells with a clear cytoplasm (Fig. 1). No mitoses were found. The scanty stroma was highly vascularized. Autopsy specimens from *the operation field contained small islands of tumour cells infiltrating* the connective and fatty tissue around the vessel transplant. The lymph node metastases consisted of tumour tissue with the same structure as that of the primary tumour.

After formaldehyde gas treatment strong fluorescence could be detected in the epithelioid cells of the carotid body tumour (Fig. 2). The intense fluorescence had the yellow-green colour typical for certain catecholamines, i.e. noradrenaline, dopamine and adrenaline. Only the cytoplasm of the cells showed specific fluorescence whereas the nucleus appeared dark. No yellow fluorescence indicating the presence of 5-hydroxytryptamine was observed.

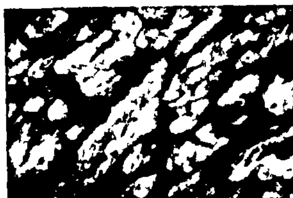


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Fluorescence microphotograph of carotid body tumour Groups of tumour cells with specific fluorescence of catecholamines can be seen surrounded with strands of non fluorescent connective tissue 100 ×

DISCUSSION

The patient described here had a malignant carotid body tumour with regional lymph node metastases The tumour contained large amounts of catecholamines in the cytoplasm Doubts have been risen about the existence of malignant forms of this tumour but well documented cases have been reported in the literature (*Moberg* 1961 *Reese et al* 1963 *Pryse Davies et al* 1964) Most of them have metastasized to the cervical lymph nodes but nearly half of them had distant metastases

It has earlier been possible to demonstrate large amounts of catecholamines in benign carotid body tumours (*Berdal et al* 1962 *Glenner et al* 1962 *Pryse Davies et al* 1964) In two instances (*Glenner et al* 1962 *Pryse Davies et al* 1964) the catecholamine present was almost exclusively noradrenaline and it may be suggested that the present catecholamine is also noradrenaline as this catecholamine was excreted in the urine It may thus also be possible that noradrenaline was released from the tumour and this may have caused the patient s hypertension Such a release can also explain the fatty change of the liver as changes of this type can be experimentally induced by catecholamines (*Carlsson & Lilyedahl* 1963)

The normal human carotid body contains two types of cells with monoamines one with a yellow green fluorescence probably containing noradrenaline and the other type with a yellow fluorescence containing 5 hydroxytryptamine (*Hamberger et al* 1966) It seems highly probable that the tumour cells in the described case originate from the cells normally containing noradrenaline

SUMMARY

A malignant carotid body tumour with regional lymph node metastases is described The tumour contained large amounts of catecholamines It is suggested that the tumour cells originate from the catecholamine-containing cells in the carotid body

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FURTHER STUDIES ON THE FINE STRUCTURE OF RENAL TUBULES IN HEALTHY HUMANS

With a Note on the Effect of the Intravenous Infusion of Low Molecular Weight Dextran

By

JAN L. E. ERICSSON, GIUSEPPE ANDRES, ANDERS BERGSTRAND, HÅRAL BUCHT and PER-AKE ÖRSTEN

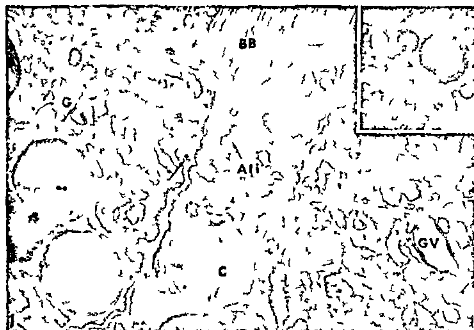
Received 26.viii.66

In a previous investigation (*Ericsson et al.* 1965a), we reported on some light and electron microscopic features of renal tubular cells in needle biopsies from young and healthy humans. It was shown that certain differences and modulations of appearance of tubular cells exist between man and experimental animals. It appeared that some of these differences were due to a less satisfactory fixation of the human material—which was obtained through percutaneous needle biopsy—as compared to the renal tissue from experimental animals, which was fixed *in vivo* by dripping (*Ericsson* 1964) or through arterial perfusion (*Ericsson* 1966a). It is apparent that those discrepancies in structure which are related to the mode of application of the fixative solution cannot be overcome, since renal tissue from living humans can only be obtained either through needle biopsy or open surgery; both of these methods seem to produce approximately the same degree and types of artifacts.

The technique of embedding in methacrylate which was used in our first study (*Ericsson et al.* 1965a) did not permit a detailed evaluation of the fine structure of the various cytoplasmic organelles, and also made this material insufficient in some respects as a basis for studies of renal tubule lesions in human material.

It was therefore considered necessary to continue our studies of the normal anatomy of the tubular epithelium in man in order to further elucidate the amount and variations of unavoidable artifacts due to the biopsy technique, and to study further the fine structure of the cell

The authors wish to gratefully acknowledge the technical assistance of Miss Cesa Thies and Mr. Lars Norman in the electron microscope and cytochemical studies.


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Figs 3 4

Fig 3 Control proximal convoluted tubule cell with apical vacuole (AV) in which the "unit membrane" structure of the bordering membrane is clearly visible (arrows) Vestopal Lead hydroxide and uranyl acetate × 66 000

Fig 4 Control proximal convoluted tubule cell containing a circular probably doughnut shaped mitochondrion (m_1) in the section completely surrounding another smaller mitochondrion (m_2) Embedding and staining as in Fig 3 × 44 600

organelles using recent developments in fixation embedding and staining techniques for electron microscopy

In the present investigation of presumably normal tubules the fine structure of tissues from biopsies processed for optimal preservation of the morphology will be described As part of an investigation of the functional and morphologic effects of low molecular weight dextran (Rheomacrodex Pharmacia Uppsala Sweden) on the kidney a report of the effect of the intravenous infusion of Rheomacrodex on renal tubule fine structure is included

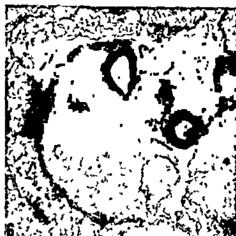
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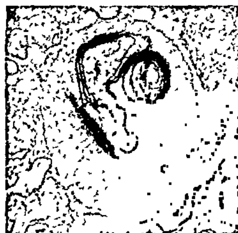
Figs 1 2

Fig 1 Control Apical portions of proximal convoluted tubule cells Note the "coated" apical tubular invagination (At) and the irregularly widened extracellular spaces between the lateral plasma membranes In one of the latter spaces is some electron dense material (arrow) present *Inset* shows thick coatings on the luminal side of 4 apical vesicles of variable size The matrix of the cytosome (C) is fairly dense BB brush border G Golgi apparatus GV Golgi vacuole Epon Lead hydroxide × 28 000 *inset* × 48 000

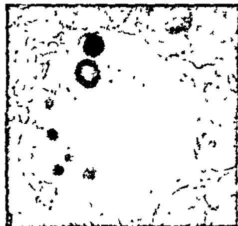
Fig 2 Control Portion of cytoplasm in proximal convoluted tubule cell with a large cytosome (C) and a cytosegresome (CS) located close to the Golgi apparatus The latter is composed of vacuoles (GV) and vesicles (Gv) Some of the vesicles are closely related to the cytosome Note the double layered envelope (arrow) of the cytosegresome which contains a well recognizable mitochondrion Epon Lead hydroxide × 32 000

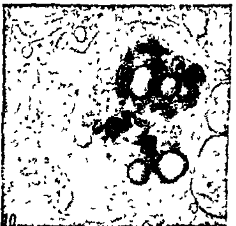












MATERIAL AND METHODS

Clinical investigations prior to biopsy included determinations of serum creatinine and endogenous creatinine clearance, studies of the sediment, and tests for proteinuria. Renal tissue for the morphologic studies was obtained through percutaneous needle biopsy as previously described (*Ericsson et al* 1965a)

For the electron microscopic studies of the appearance of normal renal tubules, small pieces of needle biopsy specimens from 4 young and healthy humans (age 17 to 30 years) lacking signs of proteinuria or impaired renal function were studied. In addition presumably normal renal tissue was obtained during surgery from the unaltered poles of the kidney in 2 cases (36 and 42 years of age) with small renal carcinomas located in the opposite pole; there were no signs of functional renal disturbance in these cases

The experimental group receiving Rheomacrodex included 7 subjects, 17 to 43 years of age with normal sediment, endogenous creatinine clearance, and serum creatinine. Four of these subjects showed trace of protein in their urine, while 3 did not have proteinuria

The subjects in the experimental group received an intravenous infusion of 1000 ml of Rheomacrodex approximately 12 hours (3 cases) and 24 hours (4 cases) prior to renal biopsy. *Infusion time was 1 hour. Free access to food and water was allowed* in both the control and the experimental groups

The biopsy specimens were rapidly divided and tissues for electron microscopy were immersed in 2 per cent osmium tetroxide (OsO_4) buffered with s-collidine (pH 7.4) at 0–+4° C. After an immersion period of 2 to 5 minutes, the specimens were fur [illegible] fixation [illegible] solutions [illegible] oxide or [illegible] tissues obtained during surgery were treated as described above. In addition, small [illegible] hours in 3 per [illegible] were utilized [illegible] rophosphatase [illegible] ubation times in the freshly prepared Gomori type medium (*Barka & Anderson* 1963) varied be-

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Figs 5–10

Figures 5 through 10 show the appearance of single membrane limited cytoplasmic bodies in the cells of proximal convoluted tubules from normal control cases embedded in Epon and stained with lead hydroxide

Fig 5 Cytosome with moderately electron dense matrix containing cytosomal dense and membranous or filamentous material (arrows) × 29 000

Fig 6 Cytosome containing membrane bound inclusions (arrows), possibly representing remnants of digested organelles, as well as thick walled irregularly shaped bodies × 39 000

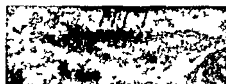
Fig 7 A cytosome (C) with myeline figure like material (parallel membranes) and a not recognizable body possibly a partially digested organelles such as a mitochondrion × 60 000

Fig 8 Single membrane limited body (CS) containing what appear to be two altered mitochondria (note crista-like structure in one of them, marked by arrow). This body is accordingly considered to be a cytosegresome × 39 000

Fig 9 A cytosome containing remnants of membranes and numerous rounded electron dense bodies similar to the ones seen in lipofuscin like organelles (*cf* Fig 10) × 36 000

Fig 10 A lipofuscin like body containing abundant rounded densities and irregular tangles of membranous or filamentous material × 30 000


12




14

tween 30 and 45 minutes Prior to thin sectioning approximately 1 μ thick sections cut with glass knives on LKB Ultrotomes were stained with alkaline toluidine blue and were studied in the light microscope Peripheral areas of the blocks showing good preservation—as judged by light microscopy—were selected for fine structural observations The thin sections were picked up on formvar and carbon coated grids and were stained with lead hydroxide (*Karnovsky* 1961) and/or aqueous uranyl acetate (*Watson* 1958) They were studied in a Siemens Elmiskop I electron microscope

For paraffin embedding and light microscopy portions of biopsy specimens were fixed in 2 per cent paraformaldehyde in 0 1 M phosphate buffer (pH 7 4) Sections of paraffin embedded material were stained with hematoxylin and eosin van Gieson s connective tissue stain and the periodic acid Schiff (PAS) method

OBSERVATIONS

Normal Subjects

A *Light microscopy* The tissues embedded in paraffin showed an appearance similar to that described previously Thus, many proximal convoluted tubules contained large amounts of "debris" while others showed collapse or formation of apical "blebs" In 1 μ thick sections of plastic-embedded material stained with toluidine blue, expanded basilar extracellular spaces were sometimes observed However, these structural modulations were not nearly as prononunced as those observed in the previous investigation (*Ericsson et al* 1965 a) Furthermore, artifactual displacement of cellular organelles to tubular lumens tubular collapse, and general distortion of the tubules was much more infrequent and less pronounced in the present material

Similar to the situation in the rat kidney, reaction product in the procedure for acid phosphatase showed a distinct localization in intracellular droplet form in all portions of the tubules except some areas

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Figs 11 14

Figures 11 through 14 illustrate the appearance—at high magnification—of different cytoplasmic organelles in control cases embedded in Vestopal With this embedding medium the substructure of bordering membranes was better resolved than with other media Sections are stained with lead hydroxide and uranyl acetate

Fig 11 Cytosome containing clumps of dense material and bordered by a triple layered approximately 100 Å thick membrane The plasma membrane (PM) is similar in thickness and appearance as the cytosomal membrane × 210 000

Fig 12 A cytoplasmic body (B) possibly a mitochondrium is completely surrounded by two triple-layered approximately 70 Å thick membranes which may represent smooth surfaced endoplasmic reticulum × 165 000

Fig 13 Portion of endoplasmic reticulum in one part studied with ribosomes (arrows) The triple layered bordering membrane is 60–70 Å thick × 230 000

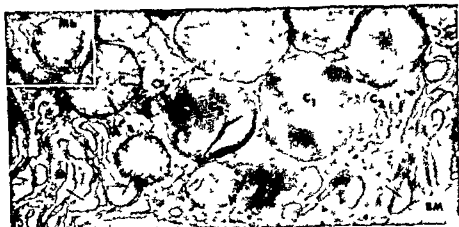
Fig 14 Portion of microbody like structure (Mb) bordered by triple layered 60 70 Å thick membrane Comparison with the cytosome in Fig 11 shows that the cytosomal membrane is thicker than that of the microbody like element (the magnification is higher in Fig 14) × 270 000

of the thin limbs of Henle The droplets were up to approximately 2 μ in diameter in the proximal convoluted tubules (Fig 17) while they were smaller and less numerous in the "pars recta", (Fig 18) and the distal portions of the nephron.

B *Electron microscopy* Since some of the features of the tubular epithelium has been described previously (*Ericsson et al.* 1965a), only those structures and modulations of appearance which were not revealed previously will be considered in the following The localization of acid phosphatase was essentially similar to that in the rat kidney (*Ericsson* 1966b) with reaction product mainly confined to cytosomes A more detailed account of the localization of acid phosphatase in the various portions of the nephron will be published elsewhere (*Ericsson & Trump* 1967), and the results are only summarized here

Proximal convoluted tubules The appearance of these tubules is illustrated in Figs 1 to 15 It was noted that the apical tubular invaginations (Fig 1) and apical vesicles (cf Fig 22) were "coated" in a similar fashion as in corresponding cells in the rat kidney (*Ericsson* 1964 1965) These structures seem to participate in pinocytosis, particularly in the transport of proteins and other colloidal compounds (*Ericsson* 1964, 1965, *Roth & Porter* 1962) Apical vacuoles (Fig 3) and vesicles were bordered by a "unit membrane" with similar dimensions as the apical plasma membrane This supports the assumption (*Ericsson* 1964, 1965) that these structures represent pinocytosis vesicles and vacuoles which have been pinched off from invaginated portions of the apical cell membrane Mitochondria varied greatly in size and appearance (Fig 4) and contained numerous large matrical granules (cf Fig 23)

Since single membrane limited cytoplasmic bodies—presumed to represent "lysosomes"—appear to be altered following infusion of dextran and other polysaccharides to experimental animals (*Janigan & Santamaria* 1961, *Maunsbach et al* 1962; *Trump & Janigan* 1962) special emphasis was placed on the elucidation of the appearance of such bodies At least three different types were observed The most frequently encountered of these bodies resembled the cytosomes in rat kidney proximal tubule cells (*Ericsson* 1964, *Ericsson & Trump* 1964) (Figs 1 7, and 9 11) they appeared to contain acid phosphatase (Fig 19) and were limited by an approximately 100 Å thick, triple-layered membrane Occasionally, bodies containing clearly recognizable cytoplasmic organelles such as mitochondria, were seen Since these bodies were observed so infrequently, it could not be demonstrated with certainty whether they too carried acid phosphatase Some of them were surrounded by two membranes (Fig 2), each with a thickness corresponding to that of the endoplasmic reticulum (Fig 12, cf Fig 13) Gradual transitions between such "cytosegresomes" (*Ericsson* 1964, *Ericsson & Trump* 1964 *Ericsson et al* 1965b) ("autophagic vacuoles") and cytosomes were encountered (Figs 7 and 8) The third type of





Figs 15 16

Fig 15 Contr l Base of proximal convolute l tubule cell show ng irregular widen ing of the extracellular spaces (arrows) between the infolded l asilar plasma membranes Three cytosomes (C_1 C and C_3) are present in the cytoplasm C_1 lacks membrane inclusions in the matrix C contains small amounts of such material wh le C_3 appears t contain cytoplasmic matrix and may correspond to a cytogresome *Inset* shows a body (Mb) presume l to repre sent a microbody it is surroun led by endoplasmic reticulum but—in the plane of the section—d es not show presence of a nucleoi l BM basement membrane Epon Lead hydroxide × 24 000 inset × 29 000

Fig 16 Control Portion of a loop of Henle with flattened epithelium The apical plasma membrane forms irregular protrusions and microvilli (Mv) Note the very thick and lamellated basement membrane (BM) Note also the filaments (F) in the cytoplasmic matrix A lipofuscin like body is present in the cytoplasm C Golgi apparatus L tubular lumen Epon Lead hydroxide × 18 000




19

cytoplasmic bodies had a finely granular matrix substance, was frequently surrounded by endoplasmic reticulum and often contained a slender peripheral rod or plate. Such bodies are illustrated in Figs. 14, 15, 22 and 23). These structures have tentatively been referred to as microbodies due to (a) the close and frequent association with the endoplasmic reticulum (*Ericsson* 1964, *Ericsson & Trump* 1966, *Novikoff & Shin* 1964, *Trump et al.* 1962), (b) the similarity of the rods to "nucleoids" in some species (*Miller* 1961, *Jones & Fawcett* 1966, *Trump et al.* 1962) and (c) the fact that the bodies were limited by an approximately 70 Å thick triple layered membrane (Fig. 14) (*Ericsson & Trump* 1966, *Maunsbach* 1966).

The Golgi zones were large (Figs. 1 and 2, cf. Fig. 22) and were often closely associated with cytosomes and cytosegresomes (Fig. 2).

In the previous study (*Ericsson et al.* 1965 a) pronounced widening of the extracellular basilar "compartments" was frequently observed. Although such widening did occur also in the present material, it was usually not very marked (Fig. 1, cf. Fig. 23). Occasionally electron dense "proteinaceous" material was observed in the widened extracellular spaces (Fig. 1).

Straight part of proximal tubules. A few observations were made of this portion of the nephron. The tubular epithelium was low (Fig. 20) with relatively sparse cytoplasmic organelles and small cytosomes. Invaginations of the basilar plasma membrane were either lacking completely or were very shallow.

Other portions of the nephron. The loops of Henle were surrounded by extremely thick and lamellated basement membranes (Fig. 16). The cytoplasm of the lining cells often contained numerous dense lipofuscin-like granules, and numerous filaments were disposed in the cytoplasmic matrix.

Two characteristic features of the cells in the collecting ducts were noted: (a) The presence of numerous randomly oriented filaments in the cytoplasmic matrix, and (b) peculiar configurations of mitochondrial cristae (Fig. 21).

Figs. 17–19

Figures 17 through 19 show the localization of acid phosphatase in a control case.

Fig. 17. Light micrograph showing proximal convoluted tubules with reaction product in "droplets" of variable size. × 640.

Fig. 18. Light microscopic picture of pars recta containing numerous small droplets. × 480.

Fig. 19. Electron micrograph showing reaction product mainly in bodies with the size corresponding to that of cytosomes. There is some spurious precipitation in the cytoplasm surrounding the bodies. Epon. Unstained section. × 24 000.

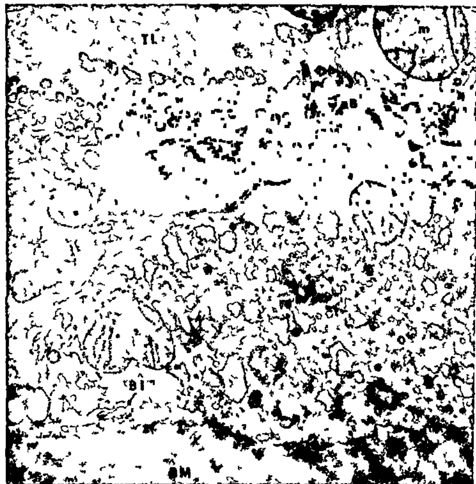


Fig. 20

Control. Section from the inner cortex showing the appearance of the straight part of the proximal tubule (*pars recta*). Cytoplasmic organelles are more sparse, the brush border (BB) is lower and the basilar invaginations (BI) of the plasma membrane are more shallow than in the proximal convoluted tubules. There is artifactual localization of a mitochondrion (m) in the lumen of the tubule (TL). BM, basement membrane. Epon. Lead hydroxide. × 21 000

Subjects Given Rheomacrodex Infusion

A. *Light microscopy.* The appearance of the proximal convoluted tubules as well as other portions of the nephron was similar to that in the "controls".

B. *Electron microscopy.* Examples of the appearance of proximal convoluted tubules are given in Figs. 22 and 23. With the exception of the occurrence of occasional single membrane limited bodies resembling "absorption droplets" (*Ericsson* 1965, *Maunsbach et al.* 1962a, *Trump & Janigan* 1962) and containing a finely granular substance, no alterations clearly at variance with the appearance in the controls were ob-

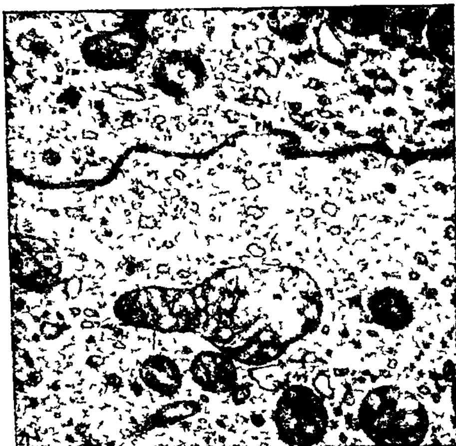


Fig 21

Control Section from the outer medulla sl owing po tions f tw cells in a c llect ing duct The cytoplasmic matrix contains al n lant haphazar lly arranged fila ments N te contin ity l etween ensel pe an l intracristal space (arr ws) in the mitochondria (m) and the angular conf gurati n and l ranching f cri tae in tl e large mit chond ion in the center C, cyto me I M pla ma meml rane Ep n l ea l hydroxide × 36 000

served Altl ough occasional cytosegresomes were encountered (Fig 22) these were not more frequent tl an in controls

COMMENT

The observations in this study have in large part corroborated previous results concerning the fine structure of the renal proximal tubules in young and healthy human subjects (*Ericsson et al* 1965a) The im proved preservation afforded in the present investigation made it pos sible to extend the early observations and to study in greater detail various types of cytoplasmic bodies and matrix materials as w ll as plasma membrane configurations



Fig. 22

Rheomacrodex infusion 24 hours prior to biopsy. Proximal convoluted tubule. With the exception of the presence of a single membrane limited "droplet" (Dr) containing a finely granular material, the apical cytoplasm shows the same appearance as in the controls. The vacuoles (V) immediately below the brush border (BB) may represent cytosomes since they contain some membraneous material. Two cytoplasmic bodies (Mb) are closely surrounded by endoplasmic reticulum and contain a slender rod. These bodies may be the equivalent of microbodies in other tissues. AV apical vacuoles. Av apical vesicle (with "coating" on the inner surface). C, cytosome containing some membranes material (arrow). CS cytosegresome, FS dilated extracellular space between lateral plasma membranes. G and G_1 Golgi zones. G is very large and contains expanded vacuoles (GV). Jc junctional complex. Epon. Lead hydroxide. × 15 000

The finding of cytosegresomes in proximal convoluted tubule cells indicates that a physiologic turnover of cytoplasmic organelles occurs in a fashion similar to that in corresponding cells of rat kidney (*Ericsson et al.* 1965b) as well as several other cell types. Although the final

proof that these organelles contain hydrolytic enzymes would require a positive outcome with ultrastructural cytochemical methods, the appearance of the bodies makes it highly probable that they are involved in the degradation of the enclosed organelles. The occurrence of apparent transitional forms between cytosegresomes and cytosomes is compatible with the notion (*Ericsson* 1964, *Ericsson et al.* 1965b) that some cytosomes are derived from cytosegresomes. The cytosegresomes appeared to be formed through wrapping of membranes—with dimensions similar to those of the *endoplasmic reticulum*—around the organelles set aside for digestion.

The microbodies in hepatic and renal tubule cells of various species appear to contain enzymes other than the hydrolases presumed to be present in cytosomes and cytosegresomes (*Afzelius* 1965, *Baudhuin et al.* 1965, *de Duve & Baudhuin* 1966, *Hruban & Swift* 1964, *Tsukada et al.* 1966). Comparisons of the appearance of microbodies in different species demonstrate that these bodies may show rather marked variations in structure (*Afzelius* 1965, *Ericsson & Trump* 1966, *Jones & Fawcett* 1966, *Trump et al.* 1962); however, they are characteristically surrounded by endoplasmic reticulum (*Ericsson* 1964, *Ericsson & Trump* 1966, *Novikoff* and *Shin* 1964, *Trump & Ericsson* 1964) and—in many instances—contain a crystalline "nucleoid" (*Afzelius* 1965). In rat kidney proximal tubules, microbodies are limited by an approximately 70 Å thick triple layered membrane (*Ericsson & Trump* 1966, *Maunsbach* 1966). A distinguishing cytochemical characteristic is furthermore their apparent lack of acid phosphatase activity (*Ericsson* 1964, *Ericsson & Trump* 1966, *Miller & Palade* 1964, *Trump & Ericsson* 1964). Since the bodies observed in the present investigation were closely surrounded by endoplasmic reticulum, were limited by a 70 Å thick membrane and some of them appeared to contain a rod shaped or plate like nucleoid, they were tentatively referred to as microbodies. Similar structures have been noted in normal human proximal tubules by others (*Bulger & Trump* 1966, *Tisher et al.* 1966). The significance of the nucleoid is not clear. In hepatic parenchymal cell microbodies of the rat the nucleoid has a crystalline appearance and may represent uricase (*Baudhuin et al.* 1965, *Hruban & Swift* 1964, *Tsukada et al.* 1966). However, a suggestive crystalline appearance of the nucleoid has also been observed in renal proximal tubules in rat kidney (*Ericsson & Trump* 1966) where uricase appears to be absent (*Graham & Karnovsky* 1965, *Straus* 1956). A non crystalline nucleoid somewhat similar to the one noted in the present investigation has been described in microbodies of hamster (*Jones & Fawcett* 1966). Since man—like uricotelic vertebrates such as birds and most reptiles—have uric acid as the principal end product in nitrogen metabolism and lack uricase (for reviews see *Afzelius* 1965 and *Florkin & Duchateau* 1943), it is unlikely that the rod or nucleoid observed in the present study represents



Fig 93

Rhe macr lex infusion 24 hours prior to biopsy. Proximal convoluted tubule. With the exception of the presence of a single membrane limited "droplet" (Dr) containing a finely granular material, the apical cytoplasm shows the same appearance as in the controls. The vacuoles (V) immediately below the brush border (BB) may represent cytosomes since they contain some membraneous material. Two cytoplasmic bodies (Mb) are closely surrounded by endoplasmic reticulum and contain a slender rod. These bodies may be the equivalent of microbodies in other tissues. AV apical vacuoles. Av apical vesicle (with "coating" on the inner surface). C, cytosome containing some membranes material (arrow). CS cytosegresome. FS dilated extracellular space between lateral plasma membranes. G and G_1 Golgi zones. G is very large and contains expanded vacuoles (GV). Jc junctional complex. Epon. Lead hydroxide. × 15000

The finding of cytosegresomes in proximal convoluted tubule cells indicates that a physiologic turnover of cytoplasmic organelles occurs in a fashion similar to that in corresponding cells of rat kidney (*Ericsson et al* 1965b) as well as several other cell types. Although the final

urease. Furthermore, it does not appear to show the characteristic crystalline appearance of urease (*Tsukada et al.* 1966).

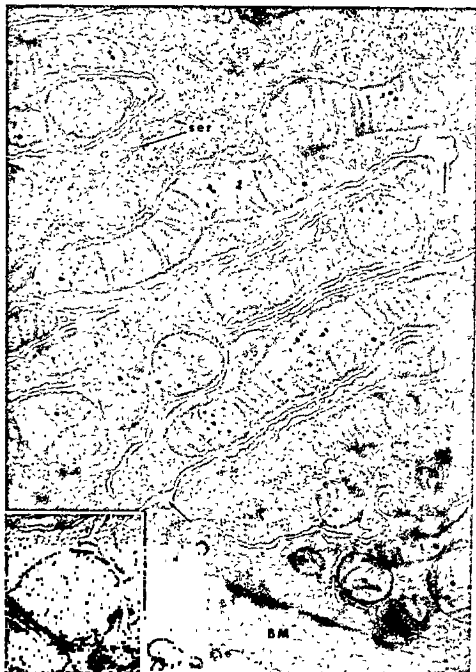
Although widened extracellular compartments in proximal convoluted tubules were noted in the present study, they were not as large and frequent as was observed previously. It is not likely that this discrepancy is related to the embedding procedure (*Ericsson et al.* 1965a). It would rather seem that the more rapid penetration of the tissues of s-collidine buffered (*Bennett & Luft* 1959)—as opposed to veronal acetate (*Palade* 1952) or phosphate buffered (*Millonig* 1961)—OsO_4 gives a more uniform and reliable fixation (*Ericsson & Klatskin* 1965, *Orrenius & Ericsson* 1966). In the rat kidney, widened extracellular compartments in proximal convoluted tubule cells are particularly prominent in a zone subjacent to the one fixed *in vivo* by dripping the fixative onto the surface of the kidney, and also in the zone below the well fixed peripheral portion of tissues fixed by immersion (*Ericsson & Biberfeld* 1966, *Maunsbach et al.* 1962b, *Trump & Ericsson* 1965). Widening of extracellular compartments can be prevented by perfusion fixation in the experimental animal (*Ericsson* 1966a). It appears to be related to the tonicity of the fixative solution as well as to increased interstitial pressure following cessation of proper circulatory conditions in the tissues (*Maunsbach et al.* 1962b, *Trump & Ericsson* 1965).

The nature and significance of cytoplasmic filaments is obscure. They have been observed in a large number of different cell types. Their morphologic appearance suggests a supportive function; they may also be associated with cytoplasmic streaming. The occurrence of numerous filaments in the cells of the collecting ducts is compatible with either of these interpretations.

Studies on experimental animals have suggested that various carbohydrates and polysaccharides which are filtered through the glomeruli are reabsorbed in the proximal tubules, probably by way of pinocytosis (*Gloor* 1965, *Maunsbach et al.* 1962a, *Trump & Janigan* 1962). It appears furthermore that these substances are transported to and stored within the cytosomes. If the absorption is excessive, the cytosomes become greatly enlarged and the light microscopic picture of "vacuolar nephropathy", "osmotic nephrosis" or hydropic degeneration ensues.

Fig. 23

Rheomacrodex infusion 24 hours prior to biopsy. Base of proximal convoluted tubule cell showing normal appearance of cytoplasmic constituents and matrix substance. Note focal expansions (arrows) of extracellular spaces between infolded basal
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NOTE ADDED IN PROOF

After this paper was submitted for publication a paper by *Tisher et al* (Lab Invest *15* 1357 1966) has been published describing the fine structure of the human proximal tubule The findings are essentially in agreement with our observations Thus the authors describe the occurrence of microbodies with rod shaped or plate formed nucleoids and they show several pictures of cytosegresomes

Plateformed nucleoids in microbodies of bovine liver were recently described by *Shnitka* (J Ultrastr Res *16* 605 1966)

Acta path et microbiol scandinav 69, 514 520 1967

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TROPHOBLASTIC AND SUBTROPHOBLASTIC MINERAL SALT DEPOSITION IN HYDRAMNIOS

By

KAI KROHN, ARNE LJUNGQVIST and BENGT ROBERTSON

Received 28 vi 66

In a previous paper, *Ivemark et al* (1962) reported two cases of generalized arterial calcification and hydramnios in stillborn infants Both cases showed deposition of hydroxyapatite in the elastic layer of large arteries and in the trophoblastic layer of the placental villi This particular type of salt deposition in the placenta is to be distinguished from the usual type of calcification in the ageing placenta, since the latter type mainly affects the stroma of degenerating placental villi

The present study was undertaken in order to find out whether trophoblastic salt deposition is a common feature of hydramnios

MATERIAL AND METHODS

Twenty four placentas were consecutively collected from cases of verified hydramnios. Ninety placentas from non hydramniotic pregnancies served as a control group

The hydramniotic group included one case from the 2nd and 23 cases from the 3rd trimester of pregnancy In eight cases there were congenital malformations, in three of these the malformation involved the digestive tract (oesophageal atresia cheilognatopalatoschisis agenesis of the upper digestive tract in a monster) Five other cases displayed isolated or combined cardiovascular pulmonary or cerebral malformations Maternal diabetes was present in two, toxaemia of pregnancy in four cases There was no instance of iso immunization Fifteen of the infants were stillborn

In the control group fifty of the placentas were consecutively collected from uncomplicated full term pregnancies the placentas as well as the infants being considered grossly normal The remaining forty placentas were consecutively collected from cases of perinatal death Two of these were from the 2nd and 38 from the 3rd trimester of pregnancy In c [illegible] of various types including one case [illegible] mations involve the digestive tract [illegible] iso immunization in eight cases Th [illegible]

For histologic examination of the placenta blocks were taken from two to five areas fixed in 10 per cent neutral formalin and embedded in paraffin Sections 3 5 μ thick from these blocks were stained with haematoxylin-eosin PAS von Kossa's stain for phosphates and carbonates and with the Murexide method which according to *Kaufman & Adams* (1957) is highly specific for calcium In addition sections were stained with Prussian blue for iron since subtrophoblastic iron deposition has been reported to occur during the second trimester of gestation (*McKay et al* 1958)

Presence of trophoblastic and subtrophoblastic deposition of calcium, iron and phosphates/carbonates was recorded separately. The amount of such deposition in each case was graded as follows:

0	No deposition
+	Deposition in less than 10 per cent of the villi
++	Deposition in 10-30 per cent of the villi
+++	Deposition in more than 30 per cent of the villi

For microradiographic examination 5 µ thick sections were cut from the formalin fixed specimens and placed in close contact with a fine grain photographic emulsion. The sections were exposed in a fine focus X-ray tube at the wave length range 8-20 Å. The microradiograms of the placentas were compared with the corresponding stained sections.

For evaluation of the statistical significance of the results Yeats' corrected χ^2-test was used.

RESULTS

Incidence and Degree of Mineral Deposition

Trophoblastic and subtrophoblastic salt deposition was found in nineteen of the twenty-four hydramniotic cases (79 per cent). Fourteen of these displayed deposition of calcium, iron and phosphate/carbonate. Three cases showed iron deposition only. In the remaining two cases there was deposition of calcium and phosphate/carbonate but not of iron.

In the control group no trophoblastic or subtrophoblastic deposition of calcium or phosphate/carbonate could be demonstrated. In five cases, all from the 3rd trimester (5.5 per cent), subtrophoblastic iron deposition was found.

TABLE 1

The Occurrence of Various Grades of Trophoblastic Mineral Deposition in Twenty-Four Cases of Hydramnios

Grade*	Per cent of cases with deposition of		
	Calcium	Iron	Phosphate/Carbonate
0	33	29	33
+	21	17	21
++	38	33	38
+++	8	21	8

* For definition see text.

The degree of salt deposition in the hydramniotic group and in the control group is presented in Tables 1-2. The difference between hydramniotic and non-hydramniotic groups as regards presence of trophoblastic salt depositions is highly significant for both calcium and iron as well as for phosphate/carbonate ($p < 0.001$). No correlation could be found between the occurrence of such mineral deposition and malformations of the foetus or obstetric complications (stillbirth, toxaemia of pregnancy).

TABLE 2

The Occurrence of Various Grades of Trophoblastic Mineral Deposition in Ninety Non Hydramniotic Cases (Fifty Placentas from Full Term Uncomplicated Pregnancies + Forty Placentas from Cases of Perinatal Death)

Grade*	Per cent of cases with deposition of		
	Calcium	Iron	Phosphate Carbonate
0	100	95	100
+	0	3	0
++	0	1	0
+++	0	1	0

* *For definition, see text*

Histologic Characteristics of Mineral Depositions

The *calcium depositions*, as demonstrated with the Murexide stain, were localized mainly in the cytoplasm and in the intercellular space of the syncytial trophoblast (Figs 1 and 3). Only occasionally were they found in the subtrophoblastic region, in or near the trophoblastic basement membrane

The distribution of villi with trophoblastic calcification was patchy in some areas most villi showed calcium deposits and in other areas no villi were affected In the main, areas with calcification were localized in the subchorionic region of the placenta This localization was particularly evident in cases with few affected villi The calcification mainly affected the small terminal and medium sized villi

In villi with trophoblastic calcium deposition there was stromal oedema and degeneration with fibrinoid changes and some necrosis Their basement membranes were thickened with PAS positive material (Fig 2) Villi without evidence of trophoblastic calcium deposition were normal, although cases of foetal death showed some stromal condensation and vascular collapse Both in affected and unaffected villi the degree of trophoblastic knotting and persistence of cytotrophoblasts was similar

The localization of the *iron depositions* differed clearly from that of the *calcium depositions* *Iron was found exclusively in the subtrophoblastic region*, in and around the trophoblastic basement membrane, as a rule underneath the calcium and phosphate/carbonate depositions (Fig 4) Villi with iron deposition were distributed irregularly and could be found anywhere in the placenta Stem villi, medium sized villi and terminal villi were equally affected Apart from iron deposition the affected villi appeared normal with no evidence of stromal degeneration

Phosphate carbonates as demonstrated with the von Kossa stain, were *mainly localized to the same trophoblastic region* as the calcium depositions (Fig 4), but were also encountered in the same region



Figs 1 and 2

Fig 1 Placental villi with calcium deposition in the trophoblastic layer. Hydramnios. Gestational age 6 months. Murexide stain × 530

Fig 2 Fibrinoid stromal degeneration and thickening of subtrophoblastic basement membrane (arrows) [illegible] placental villi with trophoblastic calcium deposition. Hydramnios, full term pregnancy, placental weight 350 g. PAS × 210

4

Figs 3 and 4

3 (Colour plate) Placental villus with triple phosphate calcium deposition (brown yellow). Polyhydramnios. Gestational age 6 months. Same case as in Fig 1. Murexide stain. Toluidine blue × 530

4 (Colour plate) Placental villi with triple phosphate deposition [illegible] plate/carbonate (black) and sulfur phosphate [illegible] deposition [illegible] blue. Polyhydramnios. Gestational age 8 months. von Kossa stain. Prussian blue × 530

as the iron depositions, i. e. in the subtrophoblastic basement membrane.

In placentas from hydramniotic cases showing depositions of calcium, iron and phosphates/carbonates, the various depositions largely occurred together in the same villi.

Microradiographic Findings

The microradiographic examination of the placentas with trophoblastic villus deposits showed radiopacity of these deposits as previously described in two cases (*Itemark et al* 1962). When compared with the staining reaction it was found that this radiopacity was evident both in cases of Murexide positive—Prussian blue negative depositions and Murexide negative—Prussian blue positive depositions. However, the radiopacity was heavier in Murexide positive instances.

DISCUSSION

The present study has revealed a high incidence of trophoblastic salt depositions in the placenta of cases with hydramnios, whereas in non-hydramniotic instances no such depositions were encountered. To judge from the staining properties of these deposits they contained calcium and phosphate/carbonate. This is in agreement with previous X-ray crystallographic examinations of such deposits in which they were shown to consist of hydroxyapatite (*Itemark et al* 1962).

Subtrophoblastic iron deposition in the normal placenta has been reported to be a feature of the second trimester of pregnancy (*McKay et al* 1958). In our control series of 88 placentas from the third trimester such deposition was seen in 5.5 per cent. To judge from the significantly higher rate (71 per cent) of this deposition in the placentas from cases of hydramnios, all except one from the third trimester, iron deposition in hydramnios seems either to persist or to be delayed in formation.

Whether the salt depositions in the placental villi seen in the cases of hydramnios in the present material may be of aetiologic significance in the development of hydramnios or are secondary to hydramnios developed on other grounds cannot be assessed. However, the deposition may well constitute a morphologic basis for decreased transplacental exchange of water between mother and foetus, which has been demonstrated in the hydramniotic state by *Hutchinson et al* (1959).

The degenerative changes in villi with trophoblastic depositions of calcium salts may be due to a decreased transport of nutritive substances through the placental barrier.

SUMMARY

Twenty-four placentas from cases of hydramnios and ninety placentas from non hydramniotic cases were examined histochemically and with microradiography for the occurrence of trophoblastic and subtrophoblastic salt deposition

Calcium deposition along the trophoblastic layer was present in sixteen of the hydramniotic cases (67 per cent), whereas in the non-hydramniotic cases no such deposition was found Degenerative changes were observed in the stroma of villi with calcium salt deposits

Iron deposition along the subtrophoblastic basement membranes was noticed in seventeen of the placentas from cases of hydramnios (71 per cent) and in five of the ninety control cases (5 5 per cent) No degenerative changes were seen in the stroma of villi with iron deposition

Carbonate/phosphate deposition was generally shown to accompany the calcium, and only rarely the iron deposition All these depositions corresponded to areas of radiopacity in microradiograms

It is suggested that the trophoblastic deposits in the placental villi in cases of hydramnios may interfere with the transfer of water and other substances through the placental barrier

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SEX DIFFERENCES IN TUMOUR GROWTH AND LYMPHOID REACTIONS IN MICE WITH EHRLICH'S ASCITES CARCINOMA

By

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Received 2[illegible].viii.66

Previous investigations have revealed that inhibition of lymphoid reactions by whole body irradiation does not alter early intraperitoneal growth of the Ehrlich ascites carcinoma (EAC) (*Thunold* 1966 a, b). On the other hand it is well known that both subcutaneous and intraperitoneal growth of homotransplantable tumours cause a general stimulation of the lymphoid tissue, though this is usually insufficient to prevent tumour growth (*Woglom* 1929, *Old et al.* 1959, 1960).

It has been shown that EAC grows better in male than female mice on subcutaneous injection (*Hartveit* 1962, 1965), but no sex differences have been demonstrated on intraperitoneal transplantation (*Thunold* 1966 b). The following experiment was planned to see if sex differences in growth of EAC could be related to morphological changes in the lymphoid system.

MATERIAL AND METHODS

Mice. Adult male and female albino mice of similar age from an outbred but closed colony were used (*Thunold* 1966 a). Five groups of 12 males (mean weight 21.4 ± 0.[illegible] g) and 12 females (mean weight 21.1 ± 0.3 g) were set up.

Tumour. The Ehrlich ascites carcinoma used was a 10 days transplant from a female mouse of the closed colony.

Experimental procedure.

Intraperitoneal tumour injection. Twelve male and 12 female mice were each given 5.6 × 10^6 tumour cells in 0.1 ml whole tumour ascites intraperitoneally.

Subcutaneous tumour injection. A second group of male and female mice received the same tumour dose subcutaneously on the back.

Seven days after transplantation all tumour bearing mice were killed by cervical dislocation. The animals were weighed to the nearest 0.[illegible] g after removal of intraperitoneal and subcutaneous tumours respectively. The total packed tumour cell volume and the cell free fluid volume were determined for the intraperitoneal tumours as described previously (*Thunold* 1966 a). [illegible] films were made at once from the tumour [illegible] and stained with Leishman's stain. Two hundred tumour cells were counted and the number of large lysed cells expressed as a percentage for each m[illegible]. The subcutaneous tumours were removed and weighed (wet weight). [illegible] were fixed in formalin and stained with haematoxylin and eosin for histological examination. [illegible] [illegible] were removed and weighed to the nearest [illegible] g. Lymph nodes were taken from the mesentery and from the neck region in mice bearing intraperitoneal and subcutaneous tumours respectively. The

lymph nodes were not weighed. Sections from all organs were stained with haematoxylin and eosin and methyl green pyronin and examined microscopically.

Control test for stimulating effect on the lymphoid system. A minced suspension of liver and spleen tissue from closed colony female mice was prepared in 0.86 per cent saline and 0.1 ml injected intraperitoneally to 12 males and 12 females. In addition a Seitz filtrate of the ascitic fluid from the tumour bearing donor mouse was prepared and 0.1 ml injected intraperitoneally into the 4th group of male and female mice. These animals were also killed on the 7th day after injection; the spleens and livers were removed, weighed and stained for histological examination as stated above.

Normal organ weight and histology. The 5th group of mice were killed on the same day, and the spleens and livers were weighed and examined histologically. Some mesenterial and subcutaneous lymph nodes were removed and examined in stained sections.

Bacteriological examination. The tumours were treated under sterile conditions. Aerobic cultures from the inoculum and some of the intraperitoneal and subcutaneous transplants were made on blood agar and serum broth. For anaerobical incubation the same media plus a thioglycolate medium (Bacto Fluid Thioglycolate Medium) were used.

RESULTS

Tumour growth and microscopy. As shown in Table 1 there was no sex difference in intraperitoneal tumour growth, while a clear difference was demonstrable on subcutaneous transplantation as the tumours in the male mice were larger than in the females ($P<0.01$).

TABLE 1

The Intraperitoneal (IP) Packed Tumour Cell Volume (PCV), the Cell Free Fluid Volume and the Subcutaneous (SC) Tumour Weight in Male and Female Mice 7 Days after Injection of the Ehrlich Ascites Carcinoma. Entries: means ± SD

Route of injection	No. and sex of animals	PCV of tumour cells (ml)	Cell free fluid (ml)	SC tumours (mg)
IP	12 ♂	1.65 ± 0.32	3.28 ± 1.21	
	12 ♀	1.24 ± 0.29	3.70 ± 1.16	
SC	12 ♂			154.9 ± 67.4
	12 ♀			67.8 ± 44.4

Cytological investigation of the intraperitoneal tumours revealed 4 per cent and 6 per cent large lytic tumour cells in male and female mice respectively. These cells were similar to those previously found in non-irradiated mice with EAC (*Thunold* 1966 a). The sex difference was not significant. Similarly histological investigation of the subcutaneous tumours showed no sex difference as some tumours from mice of both sexes showed small central areas with haemorrhage and necrosis.

Organs weights. The increase in mean spleen and liver weight following intraperitoneal and subcutaneous tumour growth is demonstrated in Fig. 1. The absolute weights were used as no significant body weight change occurred during the experimental period. On *intraperitoneal* tumour growth a significant increase in both spleen and

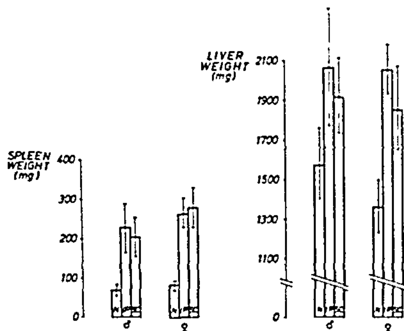


Fig 1

Spleen and liver weight in male and female control mice (N) and 7 days after intraperitoneal (IP) and subcutaneous (SC) injection of Ehrlich's ascites tumour Entries means (mg) ± SD

liver weight can be seen in male ($P < 0.001$) and female ($P < 0.001$) mice On *subcutaneous* tumour growth the increase in spleen weight was significant in both males ($P < 0.05$) and females ($P < 0.001$) The liver weight increase on subcutaneous tumour growth was similar in the two sexes ($P < 0.001$)

TABLE 2

The Average Increase (per cent) in Weight of Spleen and Liver in Mice with Intraperitoneal (IP) and Subcutaneous (SC) Ehrlich's Ascites Carcinoma

Sex of animals	Spleen wt increase (%) IP	Spleen wt increase (%) SC	Liver wt increase (%) IP	Liver wt increase (%) SC
♂	228	[illegible]	35	25
♀	231	250	46	36

The average percentage increase in spleen and liver weight is shown in Table 2 In all groups there was a tendency to greater organ weight increase in female than male mice Only the sex difference in spleen weight increase following subcutaneous tumour injection was significant ($P < 0.05$)

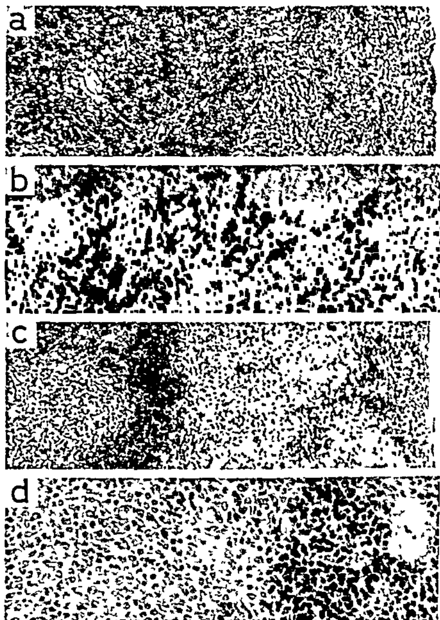


Fig 2

a Normal mouse spleen with resting lymphoid follicle (left) and red pulp Haematoxylin and eosin × 150

b Normal spleen with no distinct pyroninophilia in either follicle (left) or red pulp Methyl green pyronin × 380

c Spleen from tumour bearing mouse showing hyperplasia of the lymphoid follicle (left) The red pulp is cellular and contains a number of erythrocytes Haematoxylin and eosin × 150

d Spleen from tumour bearing mouse with large number of pyroninophilic cells in the red pulp (right) Note relative absence of pyroninophilia in the follicle (left) Methyl green pyronin × 380

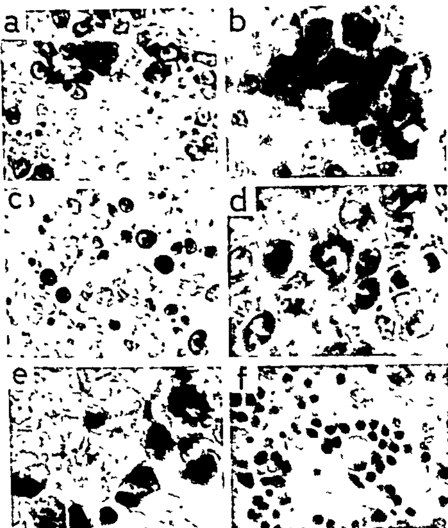
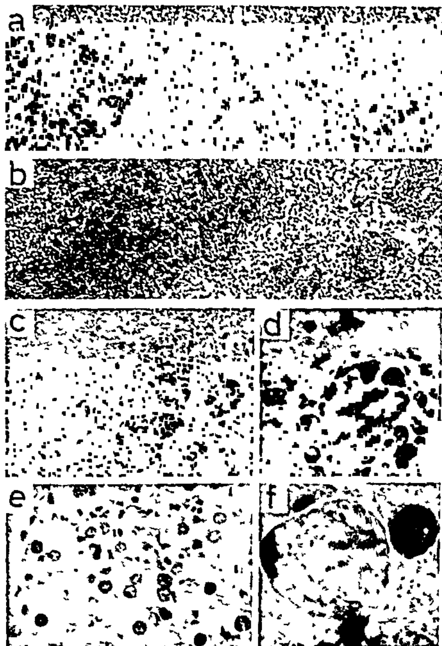


Fig 3

Spleen from tumour bearing mouse

a Red pulp with large angular pyroninophilic cells These have abundant cytoplasm and prominent nucleoli Methyl green pyronin × 610
b Greater magnification of pyroninophilic cells × 1500
c Red pulp with large pyroninophilic cells plasma cells and some lymphocytes Methyl green pyronin × 610
d Cells in mitosis from germinal centre Methyl green pyronin × 1500
e Cell in red pulp in division Methyl green pyronin × 1500
f Red pulp with large number of erythrocytes with a tendency to clumping (erythrophagocytosis?) and granulocytes Haematoxylin and eosin × 610


a
b
c
d
e
f

Organ histology In *normal spleens* the germinal centres of the Malpighian bodies contained small darkly stained cells with scanty cytoplasm and only a few erythrocytes were present in the red pulp (Fig 2 a) No distinct pyroninophilia was seen (Fig 2 b) Occasionally mast cells were present

Following both *intraperitoneal* and *subcutaneous* tumour growth the Malpighian bodies in the spleen showed prominent germinal centres (Fig 2 c) The most remarkable change occurred in the red pulp which became cellular with increased pyroninophilia (Fig 2 d) These cells were large with prominent nucleoli and abundant pyroninophilic cytoplasm (Fig 3 a b) Occasionally plasma cells were seen (Fig 3 c) There was increased mitotic activity in both the germinal centres (Fig 3 d) and in the red pulp (Fig 3 e) In addition a large number of erythrocytes with a tendency to clumping (erythrophagocytosis ?) were seen (Fig 3 f) There was no obvious changes in number or appearance of mast cells

A section from a normal *lymph node* with a resting nodule is shown in Fig 4 a On tumour growth the regional lymph nodes were enlarged with large lymphatic nodules with prominent germinal centres and cords of hyperplastic lymphoid tissue could be seen in the medulla (Fig 4 b) In the latter numbers of large pyroninophilic cells were present (Fig 4 c d)

Following both intraperitoneal and subcutaneous tumour growth red cells and granulocytes were present in the liver sinusoids (Fig 4 e) A slight increase in the size of the Kupffer cells and erythrophagocytosis could be seen (Fig 4 f)

Microscopy of the organs did not reveal any measurable sex difference

Extramedullary haemopoiesis In the normal spleen extramedullary haematopoiesis was found regularly in the red pulp Erythropoietic foci identified by the small darkly stained nuclei (*Dunn* 1954) were predominant and megakaryocytes were a constant finding Following tumour growth the erythropoiesis was more intense and the number of megakaryocytes increased (Fig 5 a b)

Fig 4

a Normal mouse lymph node with resting lymphatic nodule (left) Haematoxylin and eosin × 150

b Lymph node from tumour bearing mouse Large lymphatic nodule (left) and cords of hyperplastic lymphoid tissue in the medulla (right) Haematoxylin and eosin × 15

c Lymph node from tumour bearing m… (right) Some mast cells can be se…

d Greater magnification (× 610 … f…

e Liver tissue from tumour bearing … accumula… in the sinusoids Haematoxylin and eosin × 390

f Greater magnification (× 1 00) of Fig 4 … having erythrophagocytosis

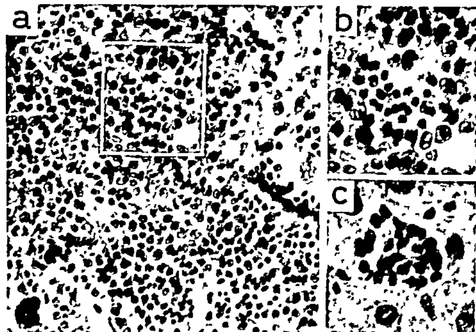


Fig 5

a Spleen from tumour bearing mouse The red pulp is crowded with large cells with pale nuclei and prominent nucleoli, and lymphocytes Several foci with erythroid elements with dark stained cells and two megakaryocytes can be seen Haematoxylin and eosin × 380
b Greater magnification of the inset in *a* showing an erythropoietic focus × 610
c Liver from tumour bearing mouse showing a sinusoid with granulopoietic elements of different maturity Haematoxylin and eosin × 610

Neither normal lymph nodes nor the lymph nodes of tumour bearing animals showed evidence of haematopoietic activity

Scattered haematopoietic elements were found in the livers of *untreated animals In this organ granulocytopoiesis was preponderant,* and megakaryocytes could not be seen After tumour growth granulocytopoietic elements were found more regularly (Fig 5 c)

Sex differences in haematopoietic activity were not seen

Control test on stimulation effect on the lymphoid tissue Neither normal mouse tissue nor a Seitz filtrate of the tumour ascitic fluid produced any increase in spleen or liver weight or give any changes *in organ histology when investigated 7 days after injection*

DISCUSSION

The present experiment confirms the previous findings that EAC grows better in male than female mice on subcutaneous transplantation (*Hartveit* 1962, 1965), while no sex differences can be demonstrated in intraperitoneal growth As a free suspension of tumour cells was used as inoculum, the number of cells given to each mouse was the

same. Thus the sex difference in subcutaneous tumour growth is likely to be due to differences in host reaction.

Following tumour growth a significant increase in spleen and liver weight is found in both sexes. The increase in spleen weight was greater in female than male mice after subcutaneous tumour injection. No sex difference could be demonstrated in either spleen or liver weight increase in the other groups.

The injected tumour was not contaminated with bacteria and there was no increase in organ weight or change in histology after injection of a cell free Seitz filtrate of the ascitic fluid or a suspension of normal mouse tissue cells. Normal mouse tissue can not be regarded as a valid control in the present experiment. It will not proliferate like tumour cells and the stimuli can thus not be compared quantitatively, even if successive injections had been given. The organ enlargement may possibly to some extent be due to the increase in extramedullary haematopoiesis but it seems in any case justifiable to conclude that the hepato-splenomegaly can be taken as an expression of host reaction against the progressively growing tumour cells. It should be stressed that the findings cannot be taken as a reaction to a tumour specific antigen in this tumour host system.

The increase in spleen weight with tumour growth is associated in the present experiment with marked histological changes in the white and red pulp of the spleen. Similar weigth and histological changes have been found with other progressively growing tumour homografts (*El Hassan & Stuart* 1965). These changes in general resemble those seen during non tumour homograft rejection (*Scothorne & McGregor* 1955, *Gowans* 1962, *Gowans et al* 1963). Thus they may be interpreted as indicating a homograft reaction to antigens present in the EAC. It has been postulated that this tumour grows due to lack of antigenicity (*Hauschka & Amos* 1957), but it has, however, been shown to contain antigens not present in normal mouse tissue (*Easty & Ambrose* 1957).

The splenic enlargement is probably partly due to accumulation of erythrocytes. It is not reasonable that this red cell accumulation is due to simple congestion. All mice, the controls included, were killed in the same way and the phenomena is also seen constantly in mice bearing small subcutaneous tumours. The possibility remains that red cells are trapped in the spleen by means of a non specific filter mechanism (see *Prankerd* 1963) or because of sensitization of the red cells (*Jandl* 1955). *Adelsberger & Zimmerman* (1954) have found that red blood cells of mice bearing isologous tumours be have similarly in the hemolytic system to mouse erythrocytes sensitized with antimouse red blood cell serum, indicating an immunological change in the erythrocytes during tumour growth. Such a possibility may also exist in the case of the homotransplantable EAC.

The change in lymph node histology during tumour growth is similar

to that found during non-tumour homograft rejection (*Ellis et al* 1950, *Scothorne* 1957) and thus gives additional evidence of immune response to the tumour homograft in the present experiment

The *origin* of the large pyroninophilic cells found in the spleen and lymph nodes has been a matter for discussion (*Gowans* 1962, *Burwell* 1962), and it has not been the purpose of the present study to elucidate this particular question The erythropoietic elements in the spleen seem to be activated during tumour growth, and it may be argued that immature erythroblasts can give a similar pyroninophilic picture However, the great number of pyroninophilic cells in the lymph nodes where no erythropoietic foci can be found and the diffuse pyroninophilia of the spleen red pulp, suggest that the pyroninophilic cells are mainly an expression of immunogical activation

While spleen and lymph node changes are striking, the histological changes in the liver parenchyma are not so characteristic It has been shown that the liver plays an important rôle in removing foreign or altered red cells from the blood stream (*Jandl & Kaplan* 1960, *Nelson & Buras* 1963) The present finding of erythrophagocytosis in the liver may support the previous suggestion of a change in red cell properties during growth of EAC

The present experiment thus gives histological evidence of activation of the immune response during both intraperitoneal and subcutaneous growth of EAC It is of interest to note the direct correlation between smaller subcutaneous tumours and greater per cent increase in spleen size in female compared to male mice The immunological nature of this stimulation may suggest that the immune response to the tumour has been stronger in female than male mice with consequent inhibition of subcutaneous tumour growth This is supported by the fact that *Andervont* (1932) found it easier to induce resistance to sarcoma 180 in female mice *Gross* (1941) demonstrated smaller incidence of takes and greater incidence of regressions of sarcoma 37 on intradermal inoculation in female mice, indicating a greater natural resistance in this sex to tumour growth In addition, it has been shown that the response to stimulating processes evoked by bacterial infection (BCG) is stronger in female than male mice (*Halpern et al* 1960)

On the other hand it has also been shown that increase in spleen size is greater in mice with progressively growing tumours than in those with receding ones (*Bashford* 1913 *Woodruff & Symes* 1962), indicating that there is not necessarily any connection between spleen size and tumour resistance A marked lymphoid reaction can be found however, in the regional lymph nodes to the regressing tumours (*Woodruff & Symes* 1962) a finding which may confirm that the seat of the reaction which destroys a first-set homograft lies mainly in these nodes (*Mitchison* 1954) It can thus be argued that the homografted tumours in these experiments (*Bashford* 1913, *Woodruff &*

Symes 1962) were destroyed before the spleen was exposed to sufficient antigenic stimulation to show histological evidence of immune activity.

No sex differences were demonstrable in either lymphoid stimulation or tumour growth during intraperitoneal growth. There is no reason to believe that the increased reactivity in the female sex should not work also in the case of intraperitoneal tumour growth. However intraperitoneal transplants of EAC usually grow fast and kill the hosts rapidly. Thus the immune response of the recipient may easily be overwhelmed and a possible sex difference in reactivity disappear. The finding of a factor in the ascitic fluid of EAC that protects tumour cells from lysis (*Harlt et al* 1961) may be of significance in this connection.

These observations may be related to the fact that some non-specific tumours give successful intraperitoneal growth while their ability to grow on subcutaneous or intramuscular transplantation is limited (*Sachs & Gallily* 1956). EAC seems to grow progressively in one hundred per cent of animals on intraperitoneal injection (*Loewenthal & Jahn* 1932, *Lettré* 1941, *Thunold* 1966 b), while *Brodersen* (1943) found approximately 84 per cent progressive growth of a solid form. It may thus be concluded that successful homotransplantation even of this highly non-specific tumour is to certain extent dependant on the site of injection and the immune response of the host.

SUMMARY

The growth of Ehrlich's ascites carcinoma (EAC) has been compared in male and female mice of similar age and related to changes in the lympho-reticular system.

On intraperitoneal transplantation no significant sex difference appeared in tumour growth, while the tumour grows better in male than female mice following subcutaneous injection.

Following tumour growth a marked hyperplasia of the lymphoid system can be found in both sexes. This hyperplasia can be related to histological changes which are of probable immunological nature, presumably evoked by tumour antigens. While intraperitoneal tumour growth showed no sex differences in these reactions, the sex difference in subcutaneous tumour growth can be directly related to a stronger host reactivity in the female sex.

It is concluded that the immune response may be of importance to subcutaneous growth of EAC. The pertinence of these observations to the fact that some homotransplantable tumours take better on intraperitoneal than subcutaneous or intramuscular injection is discussed.

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Acta path. et microbiol. scandinav. 69: 534–542, 1967

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EFFECTS OF HETEROSPECIFIC ANTISERUM AGAINST LYMPH NODE CELLS ON THE DEVELOPMENT OF EXPERIMENTAL AMYLOIDOSIS IN MICE

By

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Received 10.ix.66

In assessing the role of the immune apparatus in the pathogenesis of experimental amyloidosis in mice a number of procedures have been employed in order to modify the response of the organism to a long standing antigenic treatment leading to amyloidosis. In these experiments it has thus been shown that cortisone, when applied late in the course of amyloidogenic treatment—that is, late in or after the initial pyroninophilic phase—will enhance the formation of amyloid (*Teilum* 1952), while cortisone applied earlier in the experiment will prevent or greatly diminish the formation of amyloid deposits, mainly by preventing or postponing the pyroninophilic phase (*Christensen* 1961). Nitrogen mustard administered after cytoplasmic pyroninophilia of reticular cells has developed will even more markedly enhance amyloid deposition (*Teilum* 1954). A similar effect has been observed after whole body or spleen x-irradiation (*Christensen & Hjort* 1959, 1960). These experiments lend support to the two-phase cellular theory of local secretion of amyloid originally described by *Teilum* (1961).

In the field of experimental immunology a modified immune apparatus has been obtained by a variety of procedures such as surgical ablation techniques, ionizing radiation, or cytotoxic chemical compounds. As these methods are rather unspecific and often accompanied by undesirable, sometimes fatal, side effects, the search for a more selective immune depressant has continued and has in recent years led to the rediscovery of the leukocytotoxic serum originally described by *Metchnikoff* (1899) and the more specific antilymphocytic antiserum produced by *Pappenheimer* in 1917. Such antilymphocytic sera seem primarily to suppress immune mechanisms of the cellular or delayed type in which lymphoid cells are known to be the mediators. Recent

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This investigation was supported by grants from the *Danish League Against Rheumatism* and *[illegible] of Stenløse Peter Carl Petersen's Legacy for the Support of Medical Research*.

observations seem to imply a participation of immune mechanisms akin to the cellular type in the pathogenesis of experimental amyloidosis (*Ranløv & Jensen* 1966, *Ranløv* 1966). Further, though a cellular origin of amyloid seems well established (*Teilum* 1964, *Cohen et al.* 1965, *Werdelin & Ranløv* 1966) the exact origin of such cells and their possible relation to lymphoid cells are not known. For these reasons it was decided to investigate the effects of a heterospecific antiserum against lymph node cells on the development of casein-induced murine amyloidosis.

MATERIAL AND METHODS

Animals. Randomized, inbred C3H mice of equal sex distribution, 2–4 months old.

Antiserum. Albino rabbits weighing 3.0–3.5 kg were immunized once a week for 5 weeks with from 2 to 5 × 10^8 murine lymph node cells per injection. The cell suspensions were prepared from axillary, inguinal and abdominal lymph nodes obtained from donors of the inbred C3H strain. The lymph nodes were freed from contamination with blood and adjacent tissues, grained once in a loose fitting Potter-Elvehjem homogenizer, washed 3 times in 10 ml of Ringer's solution and resuspended in 2 ml. After cell counting and viability test with trypan blue which showed 85–90 per cent viable cells the suspension was mixed with an equal volume of Freund's complete adjuvant (Difco) and injected in multiple localizations in the rabbit. These were bled 10–14 days after the last injections. Sera were heated at 56° C for 30 minutes and stored at —20° C. As preliminary *in vivo* tests failed to show anaemia developing in the recipients no absorption with murine red cells was done. Likewise, control serum from normal rabbits (NRS) was decomplemented at 56° C and not absorbed.

Treatment. 3 groups of mice received daily 6 times a week for 3 weeks 0.2 ml of either normal saline, normal rabbit serum (NRS), or rabbit anti mouse anti lymph node cell serum (ALS) intraperitoneally. In addition, all animals received simultaneous daily injections of 0.5 ml 5 per cent sodium caseinate subcutaneously. In all animals leukocyte and differential counts were made 2–3 times weekly during the experiment. For this purpose blood was drawn from the retroorbital plexus in heparinized calibrated capillary tubes and countings were performed by standard methods. Animals dying spontaneously in the experimental period were excluded. The day after the last injection all surviving animals were killed by ether inhalation. Tissues were fixed in neutral formalin and paraffin embedded sections were stained with haematoxylin-eosin, methyl green-pyronin, the P.A.S. technique, alkaline Congo red and with thioflavin T. Amyoid was identified by its morphology and by its birefringence in Congo-stained sections when viewed under crossed polars.

RESULTS

Effects on lymphoid tissues. In the two control groups receiving casein + saline and casein + NRS no reduction in the numbers of circulating lymphocytes was seen, the average during the whole experiment being between 4.000 and 5.000 per cmm blood. In the experimental group, however, receiving casein + ALS the lymphocyte counts rapidly decreased during the first two days until stabilizing around or below 1,000 per cmm blood during the rest of the experimental period (no attempt has been made to distinguish between large, medium-sized and small lymphocytes). The average numbers of circulating mononuclear cells for each group during the experimental period are graphically

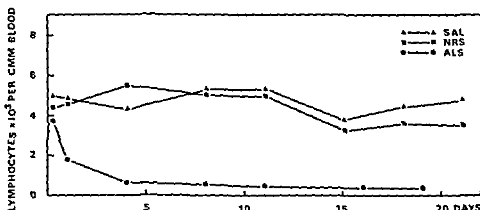


Fig 1

Mean numbers of circulating mononuclear white cells in three groups of casein treated C3H mice, simultaneously receiving intraperitoneal injections (daily) of either physiological saline (SAL), normal rabbit serum (NRS) or rabbit anti mouse anti lymph node cell antiserum (ALS)

outlined in Fig 1 After the first week a uniform transitory granulocytosis ranging from 10,000 to 25,000 per cmm blood was evident in about half the animals of each group

Lymph nodes, spleens and thymus glands from the group receiving casein + ALS were not weighed, but judged by their appearance they were not reduced in size as compared with those of the controls The lymph nodes from the two control groups treated with casein + saline and with casein + NRS, respectively, showed no histologic changes other than those usually seen developing after a protracted antigenic stimulation a general hyperplasia and increased cellularity of germinal centres In contrast the lymph nodes of the animals receiving casein + ALS showed a marked depletion of lymphocytes, the majority of cells being reticular cells and histiocytes A rather constant feature was the accumulation in the lymph nodes of histiocytes with a cytoplasm distended by a large round, bright P A S -positive inclusion (Fig 2)

Histologically the spleens of the animals treated with casein + ALS presented a uniform though rather chaotic picture (Figs 3 5). The Malpighian corpuscles were markedly reduced in number and size due

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Figs 2 5

Fig 2 Lymph node from ALS treated mouse Apart from a pronounced lymphoid depletion an accumulation of histiocytes with large bright P A S positive inclusions are seen P A S stain (× 350)

Fig 3 Severe lymphoid depletion of spleen from ALS-treated mouse Alkaline Congo red (× 35

Fig 4 Thymus and lymph node from ALS treated mouse Note the marked lymphoid depletion of the lymph node in contrast to the intact thymic cortex Alkaline Congo red (× 35

Fig 5 Spleen from ALS treated mouse Around the depleted Malpighian corpuscle many plasma cells Alkaline Congo red (× 140)




3


4



to an excessive depletion of lymphoid cells Surrounding the central artery was a loose connective tissue in which clusters of large pyroninophilic reticular cells were seen among which many plasmacytoid cells In addition a small number of mature plasma cells The splenic red pulp contained numerous histiocytes plasma cells and scattered groups of pyroninophilic reticular cells Many mitoses were seen Conversely the control spleens showed increased cellularity and large Malpighian corpuscles

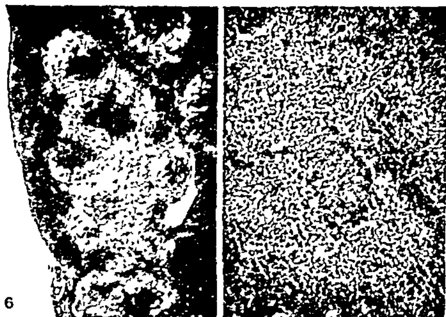
The thymus glands of the control groups showed no abnormalities In animals receiving casein + ALS the thymic structure was in all cases intact In a few animals a slight cortical depletion was noted (Fig 4)

TABLE 1

Mean Numbers of Circulating Lymphoid Cells and Mean Degrees of Spleen Amyloidosis in 3 Groups of Mice Treated for 3 weeks with either Casein + Normal Saline Casein + Normal Rabbit Serum (NRS) or Casein + Rabbit Anti Mouse Anti Lymph Node Cell Antiserum (ALS)

Treatment	Mean number of circulating mononuclear white cells during experiment (per cmm)	Number of animals developing amyloidosis Total	Mean degree of spleen amyloidosis
Casein + Saline	4 707	9/9	++++
Casein + NRS	4 529	10/10	++++
Casein + ALS	691	4/9	+

Effects on amyloid formation As shown in Table 1 only 4 out of 9 surviving animals of the group receiving ALS developed amyloid as a result of the casein treatment (among 3 more animals of this group dying spontaneously no amyloid occurred) The amount of amyloid formed in these 4 mice was far less than that of the corresponding controls ranging from (+) to + as compared to an average of + + + + in the control groups In these all mice developed splenic amyloid to about the same extent and their amyloid deposits were *found uniformly distributed as fairly regular perifollicular rings* throughout the spleens (Fig 6) However the animals receiving casein + ALS if amyloidotic showed a distribution of the amyloid quite different from that of the two control groups In some cases only 2 or 3 follicles per spleen section were involved in the amyloid formation Apart from this the sites of amyloid deposition within the spleens were similar to those of the controls the amyloid tending to stick to the perifollicular regions if a Malpighian corpuscle was almost or completely absent the amyloid would precipitate in the periphery of its remnants or around the central artery (Fig 7) In the two control groups small amounts of amyloid could be recognized in close rela



Figs 6–7

Fig 6 Spleen amyloidosis in NRS treated control, grade + + + + Haematoxylin eosin (× 35)

Fig 7 Small amounts of amyloid surrounding remnants of a Malpighian corpuscle in an ALS-treated mouse Grade + Alkaline Congo red (× 140)

tionship to the Kupffer cells lining the sinusoids of the liver and, occasionally, in the kidneys In the animals of the ALS-group no hepatic or renal amyloid could be found

DISCUSSION

In the present experiment, apart from causing severe lymphoid depletion, injections of ALS simultaneous with casein seemed to prevent the formation of amyloid in 5 out of 9 mice and markedly to reduce the degree and severity of amyloidosis in the remaining 4 mice These 4 animals also showed a different distribution of the small amounts of amyloid substance present within the spleens

The most extensive studies on the various properties of antilymphocytic sera in mice hitherto reported were those of *Gray et al* (1966) and *Monaco et al* (1966) They found that both the cytotoxic and the leukoagglutinating activities were reduced by prior absorption with spleen and lymph node cells but not with liver or kidney cells In the same way the *in vivo* lymphopenia effect could be removed Cytotoxicity was shown to be complement dependent They found ALS to be species—but not strain-specific Their findings further suggested that the antilymphocytic properties of ALS were not due to antibody pre-

sent in the ALS directed against mouse gamma globulins They found lymphopenia maintained during several weeks of treatment and for one week after, other peripheral leukocytes actually being increased The treatment with ALS was well tolerated, no weight loss or renal damage occurred Mice receiving ALS showed an elevated level of circulating cortico-steroid hormones as compared to NRS-treated controls This raise was probably a response to cell destruction by ALS rather than being responsible for the lymphopenia The histologic effects were a marked depletion of lymphoid cells while epithelial elements were preserved, complete repopulation did not occur until 6 weeks after the termination of serum therapy ALS was found to markedly suppress both first- and second-set skin homograft rejection Further, a significant inhibition of the primary humoral antibody response was observed, while no such inhibition occurred in the secondary response

Waksman et al (1961) found ALS in guinea pigs exerting a suppressive effect on each type of delayed hypersensitivity reaction presumed to be mediated by cells, the tuberculin and contact reactions were completely abolished Experimental, allergic encephalomyelitis showed a decrease in both incidence and severity and a marked delay in onset There was a similar delay in the rejection of first-set skin homografts However, the ALS had no effect on passive cutaneous anaphylaxis and the reversed passive Arthus' reaction, both mediated by circulating antibody *Nagaya & Sieker* (1965, 1966) observed only animals treated with antiserum to thymus showing depletion of thymocytes whereas thymus glands in animals receiving antiserum to lymph node cells remained intact This is to some degree in keeping with the findings in the present experiment where thymus glands of animals treated with ALS were largely unaffected in contrast to the marked lymphopenia observed in spleens and lymph nodes However the ALS-treated mice of *Gray et al* (1966) showed severe cortical depletion of the thymus *Nagaya & Sieker* found significant prolongation of skin graft survival and depression of delayed skin reaction to bovine serum albumin in ALS-treated rats In keeping with this observation are the findings of *Russe & Crowle* (1965) that mice treated with anti-thymocyte serum failed to develop hypersensitivity to antigens employed early in the experiment, or they developed the hypersensitivity with slower than normal tempo

In the present experiment and in the experiments of *Sacks et al* (1964), *Monaco et al* (1965), *Nagaya & Sieker* (1965, 1966) and *Gray et al* (1966) it was possible to maintain a persistent lymphopenia for more than 3 weeks In all the investigations cited immunologic depression was evident during the whole period of induced lymphopenia In the investigations of *Woodruff & Anderson* (1964) some animals seemingly developed long-termed or permanent tolerance to skin homografts after the termination of ALS treatment

The above mentioned suppressive effects on cellular or delayed

immune mechanisms of an immunologically induced lymphopenia correspond well with the well known immunosuppressive effects of less specific lymphopenia inducing methods in particular the use of x irradiation and cortisone In view of the similarities between the inhibitive action of both cortisone when applied in the early phase (*Christensen* 1961), and ALS on the development of experimental amyloidosis and the general immune suppressive action of both cortisone and ALS it seems justified to assume that the amyloid inhibiting effect rely primarily on an inhibition of the immune process involved in the pathogenesis of experimental amyloidosis Evidence that this immune process is in some way akin to cellular or delayed immune reactions has been collected (*Hanlon* 1966) In keeping with this assumption are the several indications cited above that antilymphocytic serum mainly acts in suppressing cellular immune responses ALS inhibition of amyloid formation may thus be considered caused by a selective suppression of immune mechanisms akin to the cellular or delayed type mediated by lymphoid cells whereas cortisone inhibition is caused by a less specific, general immune suppression Like cortisone, ALS will probably prove to exert an enhancing effect on amyloid formation if applied during the second phase of amyloid induction (*Teilum* 1952)

However, it must be borne in mind that the ALS can not be said to be truly an "antilymphocytic serum Rather is it an antiserum against a crude lymph node cell suspension containing cellular antigens representing not only small lymphocytes but also various "reticular cells The possible role of these reticular cells in amyloid formation has earlier been stressed by *Teilum* (1956 1964) On the other hand the relationship between these cells and small lymphocytes and their potential interchange under conditions of antigenic stimulation (*Gowans* 1962) are by no means clear which adds to the difficulties in interpreting results obtained with anti lymph node cell antiserum Similar experiments employing a "pure antilymphocytic antiserum prepared against thoracic duct small lymphocytes will probably provide some information regarding the cell type or types involved in amyloid formation under experimental conditions

SUMMARY

In continuation of earlier research on enhancing and inhibiting factors in experimental amyloidosis the effects of antilymphocytic serum (ALS) on the development of amyloidosis in casein treated mice have been investigated It appeared that simultaneous treatment with casein and ALS prevented amyloid formation in the majority of mice and greatly reduced its severity among the remainder

The results are discussed in view of *Teilum's* pathogenetic theory and some bearings upon the nature of the immune reaction involved in experimental amyloidosis are briefly outlined

cysteine hydrochloride, 1 per cent glucose and 5 per cent human ascitic fluid Cultivation was carried out in 500 ml round, flat-bottomed flasks filled up to the neck with medium and properly plugged The bacteria were harvested by centrifugation after incubation at 37° C for 2 or 3 days

Tests for physiological properties All tests for physiological properties were made in the enriched nutrient broth medium but the ascitic fluid content was reduced to 2 per cent Cultivation was carried out in screw cap bottles (15 ml) filled to the top with medium

Production of indol and hydrogen sulphide was tested with Kovac's reagent and lead acetate test strips, respectively

Gelatine liquefaction was tested with 12 per cent gelatine added to the fluid medium

Reduction of nitrate was examined in the enriched broth medium supplemented with 0 1 per cent potassium nitrate, as described in (2)

All tests were performed after 2 or 3 days incubation at 37° C

The fermentation studies were carried out by replacing glucose by a series of other carbohydrates (cf results), which were added separately to the sterile solutions to give a final concentration of 1 per cent The pH of the fluid media was adjusted to 7 4 After 4 days incubation at 37° C, the pH was determined by a Beckman pH meter

Serological Methods

Immunization Rabbit antisera were produced by intravenous injections of washed microbes using the dosage scheme described by *Oeding* (9) The antisera against the washed bacteria contained precipitating antibodies against the ascites component of the culture medium and had to be absorbed with ascitic fluid before use The absorptions were carried out at 37° C for 2 hours and the specimens were then left in the refrigerator overnight before centrifugation One volume of ascitic fluid was used for absorption of 50 volumes of antiserum

Ring test precipitation and *complement fixation* were performed as described in (5) Antigenic material was obtained by extracting whole microbes overnight at 37° C with 0 02 M phosphate buffer of pH 7 4

Agglutination was carried out in tubes Two drops of a saline suspension of trypsin digested microbes (turbidity corresponding to McFarland's scale no 10) were added to 0 5 ml of serum in dilutions The agglutination was read with the naked eye after incubation at 37° C in the thermostat for 20 hours

Indirect haemagglutination Washed sheep erythrocytes were sensitized as described by *Morse* (7) Extract of 200 mg bacteria (wet weight) was used for sensitization of 0 1 ml packed sheep cells The haemagglutination tests were performed as described by *Oeding et al* (10) but the haemagglutination was recorded by reading the patterns (11)

RESULTS

Morphology and Some Physiological Characteristics

The morphology and the physiological properties of the three *L. buccalis* strains included in the present study corresponded to earlier descriptions of this species The atypical filiforms differed from *L. buccalis* in their morphology, and in their capacity to produce gas and indol in fluid media (Table 1) Morphologically the atypical filiforms appeared as straight or slightly curved segmented bacilli or filaments with rounded or blunt ends The thickness and the length of the bacterial cells varied from strain to strain, and also somewhat with the cultural conditions Long filaments were particularly seen in old cultures in fluid media Young cells of the type I strains (cf Table 1) were relatively small and slender about 1 my thick and 10 my long The type II strains were more pleomorphic and varied within a greater

range from one strain to another. Both types were frequently arranged in chains. Central or subterminal swellings which did not take up a spore stain were encountered most frequently in type II strains.

TABLE 1
Morphological and Biochemical Differences between the Atypical Filiforms and L. buccalis

Strains		Morphology		Production of	
Type	Number	Bacillary	Fusiform	Gas	Indol
Atypical filiforms					
Type I	15	15	0	15	15
Type II	12	12	0	12	12
L. buccalis	3	0	3	0	0

Type I Colonies filiform and adherent
Type II Colonies varying, not adherent

The atypical filiforms showed the same lability to Gram staining as *L. buccalis*. Exponentially growing cultures incubated overnight or longer contained both Gram positive and Gram negative cells, the latter frequently with Gram positive granules. Branching was never seen. None of the strains were motile.

Colonies on blood agar of type I strains were colourless and had a tiny central eminence from which interlacing ridges radiated to filamentous edges, giving the whole colony an extremely rough and flat appearance. The type I strains were all adherent to the solid medium but gelatinous and easily emulsified. The colonies of the type II strains varied with the different strains from the filamentous appearance of the type I strains to the smooth and slimy convoluted brain shaped or Medusa head appearance of *L. buccalis* colonies. They were not adherent to the solid medium.

In fluid media the atypical filiforms grew with granular or flocculent sediment which was sometimes adherent to the side or bottom of the flasks.

TABLE 2
Fermentation Patterns of Atypical Filiforms and L. buccalis

Strains		No. of strains fermenting					
Type	Number	Galactose	Trehalose	Levulose	Raffinose	Xylose	Starch
Atypical filiforms							
Type I	5	5	5	3	5	4	5
Type II	7	7	7	7	7	6	7
L. buccalis	3	1	2	3	0	1	2

All strains fermented glucose, lactose, maltose, sucrose and mannose. None of the strains fermented mannitol, dulcitol, salicin, rhamnose, sorbitol or glycerin.

a weak serological cross-reactivity or none at all between the filamentous organisms and *L. buccalis*. Further studies are needed to clarify the taxonomical position of the atypical filamentous bacterium, although much is in favour of classifying it as a new *Leptotrichia* species, the name of which should be *Leptotrichia aerogenes*.

It is worth emphasizing the finding of antibodies to *L. buccalis* in non-immunized rabbits. Antibodies to *L. buccalis* have also been demonstrated in normal human sera (6).

SUMMARY

Some morphological, physiological and serological data concerning an anaerobic oral filamentous organism are described. The bacterium has several properties in common with *Leptotrichia buccalis*, but differs from that organism morphologically and serologically, and in its capacity to produce gas and indol in fluid media.

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Acta path. et microbiol. scandinav. 69: 549-556, 1967

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PRESENCE OF A VASO-ACTIVE FACTOR IN A NEPHRITOGENIC GROUP A, TYPE 12 STREPTOCOCCAL STRAIN

By

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Received 7.ix.66

Studies in animals concerning hypertension in experimental glomerulonephritis induced by streptococci have been reported by i.a. *Reed & Matheson* (1954a, b), *Matheson & Reed* (1959) and *Miyamoto et al.* (1960). The firstmentioned authors were able to produce nephritis and hypertension in rabbits both by infection with nephritogenic group A, type 12 streptococci and by injection of culture filtrates from these strains. From these filtrates they precipitated with ammonium sulfate a peptide which had nephritogenic and hypertensive properties. The kidney injury involved particularly the tubulus apparatus but slight or no glomerular changes were noted. *Miyamoto et al.* (1960) reported successful results in inducing typical diffuse glomerulonephritis and hypertension in rabbits infected with type 12 streptococci employing Okabayashi's paranasal sinus window method. While the triad hematuria, hypertension and albuminuria appeared after 18 to 21 days in *Reed & Matheson's* experiments, *Miyamoto et al.* (1960) noticed hematuria, oedema and hypertension after a latent period of 6 to 7 days. In all these investigations the ear capsule method was used for the registration of blood pressure. The same method was also employed by *Holm et al.* (1966) in studies concerning experimental streptococcal glomerulonephritis in rabbits. Besides a typical glomerulonephritis the latter authors were also able to produce increased blood pressure in the animals which started within a few minutes after the injection of streptococcal cell materials from a nephritogenic strain of type 12 streptococci. The possibility of the presence of a hypertensive factor in

This investigation was supported by a grant from *The Swedish Life Insurance Companies Foundation* for medical research.

We wish to thank Professor *Ö. Ouchterlony* for valuable advice and criticism during the course of this investigation. The IR spectrophotometric analyses were performed in conjunction with *Eng. Bo Skarstedt* and we want to thank him for his [illegible] *[illegible] Aller* at Chalmers Technical High School kindly [illegible] IR and UV spectra. Finally we want to [illegible] *Ag[illegible] W[illegible]nberg* for excellent technical [illegible] revising the English text.

nephritogenic streptococci was assumed The aim of the present investigation was to analyse an "autolysate" preparation from a nephritogenic streptococcal strain for the presence of a vaso-active factor and to characterize this factor from a chemical point of view

MATERIAL AND METHODS

Streptococcal autolysate A nephritogenic streptococcal strain (Group A, type 12 strain P 13846) was grown under stabilized pH conditions in an antigen free medium prepared according to the description of *Holm & Falsen* (1966) The streptococcal cells were harvested treated and subjected to autolysis as described by *Holm et al* (1966) The autolysate preparation after 10 times concentration on a Büchi Rota vapor R apparatus at 33° C was used in the gel filtration experiments

Gel filtration of concentrated streptococcal autolysate Gel filtrations were performed on 1 × 180 cm columns using Sephadex G–25 fine bead polymerized (AB Pharmacia Uppsala Sweden) A volume of 2 5 ml of the concentrated autolysate was introduced on the top of the column and eluted with newly distilled water or M/1000 phosphate buffer (pH 7 4) at room temperature The flow rate through the column was adjusted to 20 ml/hour and the material collected in 5 ml volumes by means of a siphon-controlled fraction collector (LKB, Stockholm Sweden) The optical density of each 5 ml portion was measured at 260 mμ in a Beckman DB spectrometer

Ultraviolet absorption spectrum The gel filtration eluates as well as reference substances were scanned on a Unicam SP 800 apparatus

Infra red spectrophotometry Infra red absorption patterns were obtained in a Beckman IR 7 spectrophotometer Materials to be analysed were dried over P_2O_5 mixed with KBr (1 400) and KBr discs prepared The IR patterns obtained were interpreted according to *Cross* (1960)

Animals Rabbits weighing between 1 6 and 4 5 kg were used in the animal experiments They were sacrificed between 2 and 3 weeks after the beginning of the individual experiments

Blood pressure readings The blood pressure in rabbits were measured by the ear capsule method of *Grant & Rotschild* (1934) In some experiments the intra arterial blood pressure was also recorded using a calibrated Honeywell 1508 visicorder connected via a catheter to the left femoral artery of the rabbit

Antiserum Antiserum against streptococcal material was obtained from a sheep which had been hyperimmunized with intra and extracellular material from dif [illegible] stained with hematoxylin v Gieson and examined for histopathological changes

EXPERIMENTS AND RESULTS

Gel Filtration of Concentrated Autolysate

The results of a typical fractionation experiment of the concentrated streptococcal autolysate on Sephadex G-25 employing phosphate buffer as eluent are illustrated in Fig 1 No differences in results were obtained when distilled water was used instead of phosphate buffer as effluent Six main peaks were seen when the eluted material was analysed at 260 mμ and the effluent was pooled into six fractions as indicated in the figure The fractions were concentrated to one-tenth of the fraction volume on a *Buchi Rotavapor R* at 33° C and stored at —20° C All of the antigenic material was recovered in fraction I

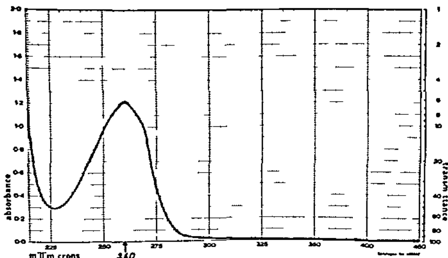


Fig 1

A typical elution diagram of concentrated strept ococcal autolysate fractionated on a 1 × 180 cm Sepha lex C 25 c lumn The shaded part of the curve represents fraction VI containing the material with vaso-active capacity

(eluted with the void volume of the column) according to diffusion in gel analysis with the antistrep tococcal serum Fraction VI was col lected after the elution of 160 ml corresponding to 4 2 times the void volume of the column Subsequent filtration of reference substances (*e g* adenine Fluka) through the same column permitted a rough estimation of a molecular weight 120 150 for materials eluted with fraction VI

The ultraviolet absorption curve of fraction VI showed a distinct maximum at 260 mμ and minima were found at 227 mμ and 287 mμ



F g 2

Figure illu trating the UV absorption curve f fraction VI The curve was obtained by scanning the material in a Un cam SP 800

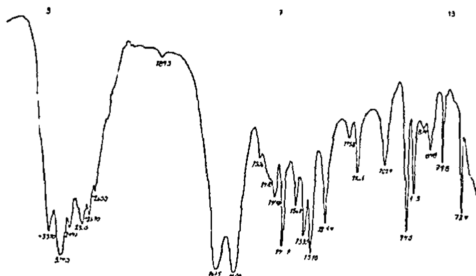


Fig 3 a

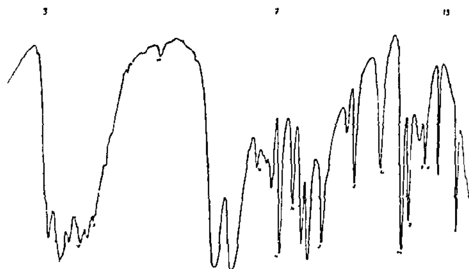


Fig 3 b

Fig 3 a and b

Infra red spectrum of a dried material from fraction VI and b adenine (Fluka). The numbers above the curves refer to the wavelength in microns. The numbers below the peaks refer to frequency in cm^{-1}, recorded on a Beckman IR 7 model

From the UV curve illustrated in Fig 2 it may also be seen that the curve was not symmetrical but showed a shoulder phenomenon at 268 mμ

Infra red spectrophotometry of dried material from fraction VI (eluted with distilled water) showed several distinct absorption maxima as may be seen in Fig 3a. Although the presence of impurities

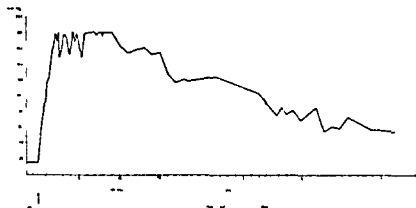


Fig 4
Diagram of blood pressure in rabbits injected with material from fraction VI The registrations were made with the ear capsule method

in the material could not be excluded the principal structure closely resembled that of adenine (Fig 3b)

Vaso Activity of Concentrated Autolysate and Fractions thereof

Investigations concerning the vaso-activity of concentrated autolysate and the six fractions obtained at gel filtration were performed in rabbits An aliquot of 0 2 ml of each preparation was injected intravenously into each of three rabbits and the blood pressure recorded by the ear capsule method Simultaneously observations concerning changes in the ear blood vessels were made After the injection of concentrated autolysate an elevation of the blood pressure was registered up to *180 mm Hg* within 2 3 min A slow decrease was then noticed during the following hours but the blood pressure was still high two weeks after the injection (60 mm Hg) The sudden elevation of the blood pressure was accompanied by a pronounced dilation of the ear vessels This dilatation slowly decreased parallel to the blood pressure during the following weeks

After injection of fractions I III no blood pressure increase was registered while fractions IV and V induced a slight elevation of the blood pressure not exceeding 45 mm Hg Only a slight dilatation of the ear vessels was observed in relation to injection of fractions IV and V

Fig 4 illustrates a typical blood pressure curve from one of the rabbits injected with fraction VI As may be seen the maximal blood pressure 180 mg Hg) was registered 2 3 min after the injection In accordance with the results obtained after injection of concentrated autolysat the blood pressure fluctuated within a wide range during the first 5 min A slow decrease was then noted during the following hours but the blood pressure was still high in all three rabbits (50–60

mm Hg) two weeks after the injection A pronounced dilatation of the blood vessels was also observed after injection of fraction VI The dilatation was maximal 2–3 min after the injection and then slowly subsided parallel to the decrease in blood pressure

An additional two rabbits were injected intravenously with 0 2 ml adenine in saline (Fluka 0 1 mg/ml) No dilatation of the ear arteries was observed and the blood pressure as measured by the ear capsule method remained within normal limits throughout the two week experimental period

The Effect of Fraction VI on Intra Arterial Blood Pressure

To investigate the observed vasal reactions the following experiments were performed Two rabbits were anesthesized with Nembutal and the left femoral artery was dissected free The artery was then connected with a Honeywell visicorder via catheter and the initial systolic blood pressure (135 150 mm Hg) was recorded Simultaneously, blood pressure readings were made with the ear capsule method After intravenous injection of 0 2 ml of material from fraction VI no elevation of the blood pressure could be registered in the femoral artery during the following two hours while with the ear capsule method an increase from the initial 20 25 mm Hg up to 140 mm Hg was noted within a few minutes followed by a slight decrease during the next two hours

Histopathological Examinations

Kidney sections from all rabbits injected with material from fraction VI were analysed histopathologically No signs of kidney lesions could be detected

DISCUSSION

In reports concerning experimentally induced streptococcal nephritis *Reed & Matheson* (1954a b) *Matheson & Reed* (1959) *Miyamoto et al* (1960) *Holm et al* (1966) have reported increased blood pressure in rabbits as registered by the ear capsule method in addition to kidney lesions The hypertension was however obtained at different times *Reed & Matheson* (1954a b) reported a gradual development of the hypertension similar to that noted with glomerulonephritis in humans *Holm et al* (1966) observed hypertension within a few minutes after the injection of streptococcal material It may be assumed that two independent factors exist in streptococci one being primarily nephrotoxic and the other being responsible for the immediate elevation of blood pressure As earlier reported by *Jonsson & Holm* (1964) similar effects could not be produced by a non nephritogenic type 7 streptococcal strain

The present results indicate that a "hypertensive factor could be

separated by gel filtration from the autolysate preparation (fraction VI) That this fraction was free from precipitinogens and lacked nephrotoxic capacity was also shown Using reference substances like adenine the molecular weight of materials eluted with fraction VI was roughly estimated at 120-150

Although the IR spectra for adenine and the material eluted with fraction VI were similar the presence of impurities in the latter material cannot be excluded However, the amount of impurities should be very small as judged by the distinct peaks in the spectrum The shoulder phenomenon at 268 mμ in the UV curve of material from fraction VI (not present in the UV curve of adenine) should then represent an impurity with an unusual high molar extinction equal to that of adenine and adenine derivates It must therefore be assumed that the material in fraction VI contains adenine and chemical compounds similar to adenine, or adenine and a hitherto unknown substance with an extremely high molar extinction, the latter suggestion being improbable As pure adenine lacked a "hypertensive" effect in rabbits, as measured by the ear capsule method, it may be concluded that the "hypertensive activity of fraction VI is most probably caused by a substance similar to although not identical with, adenine

Intra arterial recordings of blood pressure in anesthesized rabbits simultaneous to blood pressure readings by means of the ear capsule method showed that a blood pressure increase as registered by the latter method did not correspond to an elevation of central blood pressure From this experiment it may thus be concluded that fraction VI contained a substance with vaso-dilatative capacity simulating blood pressure increase noted with the ear capsule method but devoid of central hypertensive effect Whether this effect in rabbits is only localized to ear arteries or may also involve other vessels *e g* those in the kidney, cannot be stated at present If the smaller kidney arteries or even the glomerular capillaries should react in a similar way this might offer a new explanation for the location of streptococcal nephritotoxic products in the kidneys in the natural course of human glomerulonephritis

SUMMARY

A substance with a low molecular weight was separated from the precipitinogens in an autolysate from a nephritogenic, type 12, streptococcal strain by gel filtration through Sephadex G 25 Intravenous injection of the substance into rabbits resulted in a pronounced dilatation of the ear arteries and elevated blood pressure which were observed with the ear capsule method However no elevation of the central blood pressure (A femoralis) was noted The streptococcal substance responsible for the vaso activity is most probably an adenine-derivate and was found to lack nephrotoxic capacity in rat

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Acta path. et microbiol. scandinav. 69: 557–566. 1967

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BODY TEMPERATURE, ANTIBODY FORMATION AND INFLAMMATORY RESPONSE

By

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Received 3.viii.66

In 1897 *Widal & Sicard* (1) showed that poecilothermic animals were good antibody producers at high temperatures, as for instance 37° C. Antibody production was reduced at temperatures between 21° C and 33° C and completely abolished at 12° C. Similar results were achieved by *Nybelin* (2) in 1935.

In a preliminary report *Waaler* (3) showed that hibernating hedgehogs with a body temperature of 6° C did not produce antibodies and showed no local inflammatory reaction whereas they gave an active response when they were awake and warm with a body temperature of 33° C.

The ability to produce antibodies has thus been quite thoroughly studied in experimental animals, but few have paid any attention to the influence of body temperature on the local inflammatory response. This problem has been studied during hypothermia under anaesthesia in a series of papers by *Svanes* (4). He found that warm-blooded animals showed a marked reduction in local inflammation when the body temperature was reduced to 20° C–22° C.

In this communication we have repeated the experiments from 1962. As experimental animals we have used hedgehogs and also fish: cod and gold-fish. We wished to avoid the nonphysiological condition which occur in warm blooded animals during hypothermia under anaesthesia. In poikilothermic animals such as fish for instance, it is easy to change the temperature of the surrounding medium in order to produce variations in body temperature, and the condition of the experimental animal should be physiological, at least more so than during hypothermia and anaesthesia in warm-blooded animals. Hedgehogs are particularly easy to study from this point of view. In the summer season they have a body temperature of 32°–33° C. If they are placed in a refrigator at a temperature of 2–6° C they go to sleep and the body temperature drops to around 6° C. They can stay in hibernation for 3–4 months, but wake up if they are taken out of the refrigator and placed in a room with a temperature of 18°–20° C. The body temperature then goes up to values

normal in the non-hibernating state As a rule the animals will stay awake in winter if they are kept at ordinary room temperature and are fed regularly. They may however go to sleep for shorter or longer periods and stay in a "sub-hibernating state" with a body temperature of between 18°–26° C As a rule it is easy to get adequate controls

MATERIAL AND METHODS

Hedgehogs Altogether 18 hedgehogs were used, 9 non hibernating and 9 in the hibernating state In order to procedure hibernation the animals were placed in a refrigator, and the body temperature of the animals was checked at regular intervals

Inflammatory response 2 animals in each group were injected with 0 2 cc turpentine intraperitoneally The animals were killed after 24 hours The local inflammatory response was studied macroscopically and in smears

7 animals in each group were injected subcutaneously with 0 5 cc of an emulsion of silica (2 per cent emulsion in normal saline, particle size 5 micron) In 5 animals in each group the injected area was found at the end of the experiment (3 months —4 5 months) and adequate histological examination of the material could be carried out

Antibody formation Two of the experimental animals in each group in the last experiment were given parathyphoid B vaccine and washed sheep red cells intraperitoneally The animals were given 4 6 intraperitoneal injection in the last month of the experiment The hibernating animals had then been sleeping for 3 months The last injection was given one week before the termination of the experiment and blood was taken for agglutination and complement fixation tests

Fish The local inflammatory response following the intramuscular injection of 2 per cent silica in 0 15 per cent saline was studied in cod and gold fish Only pilot experiments were performed as one animal in each group was examined The cods' temperatures were 18° C for the warm and 7° C for the cold In the gold fish the temperatures were 22.5° C and 8° C respectively The warm cod did not thrive and died after 7 days whereas its cold counterpart was active and healthy In the same way the cold gold fish did not stand the low temperature well and died after ' days whereas the warm showed normal activity Apparently these animals are too sensitive to great variations in temperature, and we have been too radical in our experimental procedure

TABLE 1

Antibody Formation in Hedgehog

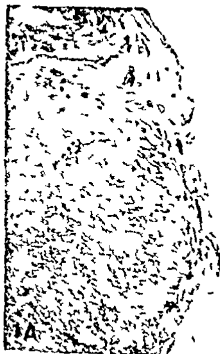
Animal No	Sheep cells		Animal No	Para B	
	aggl	compl fix		O aggl	H-aggl
1	80	80	6	572	572
2	80	80	7	64	572
3	0*	0*	8	32	0§
4	0	0	9	not tested	

No 1 2 6 and 7 non hibernating No 3 4 8 and 9 hibernating
* Highest concentration tested 1 4 § Highest concentration tested 1/10

Figs 1 2

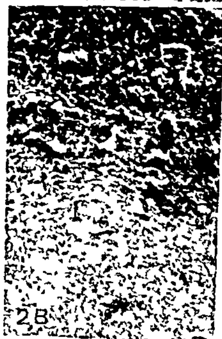
Figs 1 A and B Silica nodule in warm non hibernating hedgehog Solid granuloma with macrophages H E. A × 35 B × 140

Figs 2 A and B Silica nodule from a hedgehog in the "sub hibernating state" Peripheral infiltration of lymphocytes and macrophages Central area few cells H E. A × 35 B × 140


1A


1B


2A


2B

RESULTS

Antibody Formation in Hedgehogs

Our results are summarized in Table 1. The difference between the two groups is evident. Active antibody formation in the warm non-hibernating animals, no or insignificant activity in the cold hibernating group. Reading of the paratyphoid O agglutination was difficult. Apparently the animals showed some normal O agglutinins. Further examination and evaluation of this doubtful finding is necessary.

Local Inflammation in Hedgehogs

Our first experiment with acute inflammation after turpentine injection showed no definite difference between the cold and the warm hedgehogs. There were possibly not so many polymorphnuclears in the smears from the sleeping hedgehog as compared to the warm animal but the difference was insignificant. However, the hibernating animal was apparently not asleep at the end of the experiment. A new trial was therefore carried out with temperature control. We found that during the experiment the temperature of the hibernating animal increased to above 30° C and an adequate control could therefore not be run. This inflammatory stimulus is too great because it interferes with the hibernation. For this reason we selected silica emulsion for our next experiments.

TABLE 2

Local Silica Inflammation in Hedgehogs

Animal No	Granuloma formation	Condition of animal	
1	*Solid granuloma with macrophages*	Awake	Temp 32° C
2	Peripheral cellular reaction with macrophages. Centre: Few cells	Partly sleeping	Temp 18°-20° C
4	No cellular reaction	Hibernating	Temp 6°-8° C

Duration of experiment 5 months

Silica Granulomas in Hedgehogs

Our results are summarized in Table 2. We got three types of reaction. The non-hibernating animal (No 1) showed a solid granuloma with macrophages from the periphery to the centre (Figs. 1 A and B).

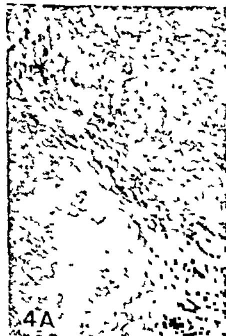
Figs. 3-4

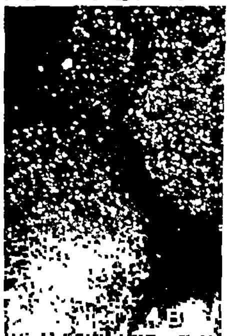
Figs. 3 A and B. Silica nodule from a hibernating animal. No cellular reaction. H.E. A × 35, B × 140.

Fig. 4 A. Silica nodule from a hibernating animal. No cellular reaction. H.E. × 150. *B.* Same field in polarized light showing the silica granules within the nodule.


3A


3B


4A


4B

RESULTS

Antibody Formation in Hedgehogs

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Figs 3 4

Figs 3 A and B Silica nodule form a hibernating animal No cellular reaction H E. A × 35 B × 140

Fig 4 A Silica nodule from a hibernating animal No cellular reaction *H E. × 150* *B* Same field in polarized light showing the silica granules within the nodule

One animal (No 2) was partly awake and partly in a sub hibernating state with varying body temperatures below the normal for non hibernating animals. It showed a granuloma with macrophages and lymphocytes at the periphery and few cells in the central area of the nodule (Figs 2 A and B). Temperature control was only carried out in the last part of the experimental period and the animal probably had a "normal" temperature in the first part.

The hibernating animal (No 4) showed a completely different histological picture. The silica remained in a definite nodule with hardly any cellular reaction (Figs 3 A and B). The experiments were repeated with similar results. This time no animal went into any kind of sub hibernating state. Silica was easily demonstrated in the nodules in polarized light. The nodules with the double refracting granulomas were clearly demonstrated in the hibernating animals and showed no infiltration of macrophages. Only a few fibroblasts were seen in the periphery surrounding the nodules as a thin capsule (Figs 4 A and B).

Silica Granuloma in Fish

The warm gold fish and the warm cod showed a definite cellular reaction with infiltration of lymphocytes and macrophages and proliferation of a few fibroblasts (Figs 5 A and B Figs 7 A and B). The accumulation of doubly refracting silica granules within the inflamed area is nicely demonstrated. The cold animals on the other hand revealed only minor degenerative changes and no inflammation. The silica granules are seen in the non inflamed areas (Figs 6 A and B Figs 8 A and B).

DISCUSSION

It is thus evident that formation of antibodies can not be demonstrated in the hibernating state. Apparently the reticulo-endothelial system is also sleeping but several questions have to be answered. What happens to the antigen during hibernation? Does it come into contact with the antibody producing organs? Is it possible that such contact in the hibernating animal may initiate a more rapid production of antibody when the animal wakes up? However these animals do not thrive and breed in captivity and it is not easy to get enough animals for experimental purposes.

Further it is evident that the low body temperature in the hibernating animals and in the gold fish and the cold cod does not permit a com

Figs 5–6

F

F


7A


7B


8A


8B

plete inflammatory response. There is a more or less complete lack of cellular infiltration and the reaction of the capillaries and the connective tissue is also absent or insignificant. Our experiments with silica do not elucidate to what extent the pure exudative processes are affected or the capillary permeability altered. Our experiments with turpentine do not throw any light on these questions either. This strong agent forces the animal to leave hibernation and increase its temperature. The animals should be examined at shorter intervals. Dilution of the turpentine and subcutaneous injection might also help us.

All normal physiological processes run at a very low level during hibernation and thus absence of response is therefore to be expected. The blood circulation is minimal and so also are the capillary bed and the oxygen consumption. The low temperatures do not give the vasoactive enzymes, so important in inflammation, the conditions necessary for their usual activity. We cannot expect marked dilatation or increase in permeability when the body temperature is 6° C. The whole complex of problems which are connected with the hibernating state is nothing but a physiological wonder, one of Nature's strange miracles. We should think that the study of some simple pathological phenomena may to some extent throw light upon the normal physiology of hibernating animals.

With regard to our few experiments with fish we are somewhat puzzled. It was to be expected that the warm gold fish should show a marked inflammatory response, whereas the cold one showed hardly any reaction. The optimal temperature of the gold fish is above 20° C. It was therefore quite natural that the cold gold fish did not respond as the animal was unable to adapt itself and died after 5 days. With the cod we found just the opposite. It is striking that the warm cod at 18° C should give an inflammatory reaction and not the cold one. The warm cod was unable to thrive and died after 7 days, but still it was able to react to the inflammatory stimulus. The cold cod was in an aquarium with a temperature which should be fairly "normal" for this fish, and the fish was active and lively. Why should the capillaries, the connective tissue, the enzymes of this fish not react at this "normal" temperature when the animal shows an active and normal behaviour? Nature has its tricks which sometimes may be difficult to understand and from a patho-physiological point of view the problem needs a more penetrating approach.

F gs 7 8

F g 7 A S lica n dule in warm c d showing s me ¤e l ma infiltrat ng f l mph
a l n l mor h nucl ars H E × 1 0 *B* Same field

F ■ ■ ■ alm t n cellu
■ 1ɔ

SUMMARY

1 Hibernating hedgehogs with a body temperature of 6° C show no production of antibodies as contrasted to non hibernating animals with a body temperature of 32° C–33° C
2 Hibernating hedgehogs and cold cod and gold fish show no local inflammatory response after injection of silicaemulsion The reaction of the warm controls was marked
3 The influence of the body temperature upon the activity of the reticuloendothelial system the vaso active enzymes and capillary permeability is discussed shortly

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Acta path. et microbiol. scandinav. 69: 567-575, 1967

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

3. *On the Homogeneity and Structure of Protein A*

By

ARNE GROV

Received 23.ix.66

The *Staph. aureus* "group antigen" *protein* A (antigen A), which produces a precipitation line on agar gel diffusion against all normal human sera, has been shown to be of protein nature (6, 11, 12).

Serologically, our protein A preparation shows two precipitation lines against rabbit immune serum and the ability to sensitize tanned sheep erythrocytes. Chromatography and electrophoresis revealed no sign of heterogeneity (6, 13). The results of absorption experiments and chemical modifications of functional groups (4) strongly supported our suggestion that the different serological activities are due to separate molecular entities of the preparation.

The present paper deals with further investigations on the homogeneity of the protein A preparation, and the molecular size and shape of entities giving rise to the serological activities.

MATERIALS AND METHODS

Antigen preparations. The protein A preparation used was that described in (6). In addition, a cold water extract was prepared according to *Löfkvist & Sjöquist* (12). After precipitation with acid, the supernatant was neutralized and subjected to serological tests.

Reagents. 2,4-dinitro-1-fluorobenzene (DNFB) was obtained from Merck, carboxypeptidase-A (toluene water suspension) from Sigma Chemical Company, crystalline



... dimension of the ... 45 to X 80 cm and a constant voltage of 500 V was used. Buffers of various pH values, all with ionic strength of 0.1, were employed, and the time for each run was 3 hrs.

Pevikon block electrophoresis was carried out as described in (2) using Pevikon C-870 and a plexiglass tray of 27 × 15 × 0.7 cm. A constant voltage of 300 V was

The ultracentrifugation experiment was carried out at *The Department of Biochemistry, University of Bergen*, for which I am very much indepted to Dr. *Karen Helle*.

supplied for 20 hrs giving a current of about 40 mA. Pieces of paper and Pevikon one cm in width were cut off and extracted with distilled water. The extracts were then tested for serological activity.

Ultracentrifugation. Sedimentation velocity, approach to equilibrium and diffusion were studied by means of the Spinco Model E analytical ultracentrifuge. A capillary type synthetic boundary cell was used in the diffusion experiment and for the approach to equilibrium run a double sector cell was used. All the ultracentrifugation experiments were performed at 20° C. The rotor speeds were 59 780 RPM in the sedimentation velocity studies, 42 040 RPM in the approach to equilibrium run and 10 589 RPM in the diffusion study. Solvent density was determined by weighing measured volumes (Agla microsyringe) of solvent and water in weighed capped bottles at 20° C. The partial specific volume of the protein was calculated from its amino acid composition (16). In these experiments the concentration of protein A was 5 mg per ml in 0.05 M phosphate buffer pH 7.4.

Digestion with trypsin and carboxypeptidase was performed as described in (4).

Free amino groups were determined by reaction with DNFB according to the description of *Ghuysen et al.* (3). On determining free amino groups after tryptic digestion the same amount of enzyme was added to the blank as was used for digestion.

Quantitative determination of amino acids released by carboxypeptidase was carried out by letting protein A (5 mg) react with the enzyme (0.25 mg) for 24 hrs at 37° C (4). Thereafter the mixture was shaken for 2 hrs with a cation exchange resin (200 mg Amberlite IR 120). The supernatant protein solution was withdrawn and the resin was washed twice with water. The pH of the protein solution with washings added was adjusted and retreatment with carboxypeptidase performed. The combined washed ion exchange resin was then extracted for 2 hrs and washed twice by shaking with aq. 5N NH_3. The extract with washings added was evaporated to dryness *in vacuo* over KOH pellets. The residue was dissolved in water and subjected to qualitative and quantitative paper chromatographic analyses as described in (5).

Treatment of immune serum and normal human serum with mercaptoethanol was performed by leaving a mixture of equal volumes of serum and 0.3 M 2-Mercaptoethanol (in saline) at room temperature for 24 hrs. In one series of experiments a 10 per cent molar excess of iodoacetamide was added to the reaction mixtures at 4° C for 1 hr, followed by dialysis against saline for 48 hrs. The treated sera were tested serologically. Sera mixed with saline instead of mercaptoethanol were always included and serum from a patient with infectious mononucleosis containing heterophile antibodies in high titre was included as control for the activity of mercaptoethanol.

Serological methods. Rabbit immune serum against the homologous strain Cowan I was produced as described by *Oeding* (14). The human serum used in this experiment was a pool of 10 normal human sera. The *ring test* and *agar gel precipitation* tests were carried out as described in (8) and (9) respectively, and the indirect *haemagglutination* test essentially as described by *Morse* (13) using a 1: 40 000 solution of tannic acid for tanning the sheep erythrocytes.

RESULTS

Serological examinations of the extracts from paper electrophoresis of our protein A preparation showed no separation of the activities. Neither was any separation achieved by Pevikon block electrophoresis in citrate phosphate buffer at pH 4.4. On paper electrophoresis it was observed that all the serologically active material moved slowly towards the cathode at pH 8.0 and slowly towards the anode at pH 8.6 indicating that the isoelectric point of protein A is between these pH values. At more alkaline and more acid pH values the migration rate increased. Serologically active material was found in a rather broad band (5 to 6 cm) and some variation in the titres of the eluates was observed.

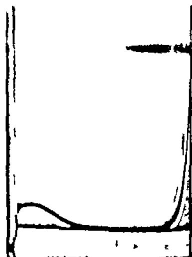


Fig. 1
Schlieren pattern of protein A (5 mg/ml in 0.05 M phosphate buffer pH 7.4) in the ultracentrifuge 161 mins after reaching 42 040 RPM

During ultracentrifugation of protein A the Schlieren patterns showed a very slow migration. The observed single sedimentation boundary indicates homogeneity of the material (Fig. 1). From the experimental results the sedimentation coefficient ($s_{20,w}$) is estimated to 1.22 at 5 mg protein A per ml. A run with a concentration of protein A of 3.5 mg per ml gave and $s_{20,w}$ of 1.26 indicating higher value at infinite dilution. The diffusion coefficient ($D_{20,w}$) estimated from the diffusion experiment at 5 mg per ml of protein concentration is 14.966×10^{-7} cm^2/sec and the partial specific volume (V_{app}) was calculated from the amino acid composition to give a value of 0.725 mg/ml. These data yield a value of 1.2 for the frictional ratio f/f_0 indicating a slightly assymmetrical molecule (19).

The approach to equilibrium run according to *Trautman* (17) gives a slope of 3.288×10^{3} and an average molecular weight (M_{app}) of 12 200. In the sedimentation study the apparent diffusion coefficient (D_{app}) increased with time (i.e. spreading of the boundary), demonstrating some degree of molecular heterogeneity (16). The increase of the diffusion coefficient was found to be 1.703×10^{-11}, i.e. a small increase. The heterogeneity could not be detected by the approach to equilibrium centrifugation as the Trautman plot gave a straight line (17).

Based on a molecular weight of 12 000 the calculation of the number of amino acid residues gives a molecular composition as listed in Table 1. The total number of amino acid residues listed is 126 giving a molecular weight of 12 021.

TABLE 1
Amino Acid Composition, integral Residues and Molecular Weight of Protein A

Amino acids	Amino acid residue (g/100 g dry protein)	Nearest integral number of amino acid residues for 12 000 g protein	Integral number of residues × respective residue molecular weights
Lys	15.6	15	1920
Asp A	14.8	15	1725
Glu A	14.7	14	1806
Ser	5.9	9	783
Gly	12.3	26	1482
Ala	9.5	16	1136
Pro	12.3	15	1455
Val	6.5	8	792
Leu	7.4	8	904
H_2O *			18
Total	99.0	126	12021

* The molecular weight of water is added to correct for the mole of water on the terminal amino acids

The total amount of free amino groups present in our protein A preparation was estimated to 1.35 μmole/mg, corresponding to 16 free amino groups per molecule (M_W 12,000)

Tryptic digestion increased the total amount of free amino groups to 2.59 μmole/mg Assuming a molecular weight of 12,000, the increase of free amino groups due to tryptic digestion corresponds to 15 amino groups per molecule

TABLE 2
Amino Acid Composition and Integral Residues of the Part of Protein A which was Digested by Carboxypeptidase

Amino acids	Amino acid residue (g/100 g dry protein)	Nearest integral number of amino acid residues for 12 000 g protein
Lys	1.3	1
Asp A	0.8	1
Glu A	0.9	1
Ser	0.7	1
Gly	0.5	1
Ala	1.2	2
Val	1.5	2
Leu	2.1	2
Total	9.0	11

After two consecutive digestions by carboxypeptidase no further release of amino acids was obtained The quantities of amino acids released are listed in Table 2 In all, only about 9 per cent of the material was digested by this enzyme The molar proportions of the

released amino acids lysine, aspartic acid, glutamic acid, serine, glycine, alanine, valine and leucine were 1.0, 0.7, 0.7, 0.8, 0.9, 1.7, 1.6, 1.8 respectively. In the same order the nearest integral number of amino acid residues should be 1, 1, 1, 1, 1, 2, 2, 2 based on a molecular weight of 12,000.

Whereas protein A gives a characteristic precipitation line on agar diffusion with all normal human sera, serum from a patient with agammaglobulinemia gave no line. This indicates that the normal serum component is a γ-globulin. Owing to scarcity of the patient's serum no other serological tests were performed. In the serological reactions with protein A and sera treated with mercaptoethanol no difference could be observed in the precipitation experiments. Treated human serum as well as treated immune serum produced the same lines as untreated sera against protein A on agar gel diffusion. Neither was any reduction of the ring test titres observed after treatment. The indirect haemagglutination test was still positive but the titres were reduced to about 1/16 of those of untreated sera. Whether or not iodoacetamide was added to the mercaptoethanol treated sera made no difference to the serological reactions.

The crude cold water extract showed the same serological activities as our protein A preparation (6).

DISCUSSION

Although a single sedimentation boundary was observed during ultracentrifugation, some heterogeneity of our protein A preparation was demonstrated by the increase of Dapp (16). The Trautman plot which gave a straight line indicates, however, that the heterogeneity is small (17), probably due to minor molecular variations within the preparation. The broad zones of serologically active material found by electrophoresis and the variation in the titres of the eluates may also indicate a certain degree of heterogeneity.

As our protein A preparation exhibits three separate serological activities, the possibility of at least three different types of molecules exists.

The sensitizing ability of protein A is the only activity lost by carboxypeptidase treatment (4). The small part of the preparation digested by this enzyme thus seems to be responsible for the sensitizing ability. Assuming complete digestion, the sensitizing substance constitutes a maximum of 3 per cent of the total weight. The major part of the material should then constitute the precipitinogens which apparently are open polypeptide chains as indicated by their content of simple amino acids and their relative stability to heat.

The slow sedimentation of protein A in the ultracentrifuge rendered the measurement of the sedimentation coefficient somewhat uncertain. Good agreement was, however, obtained for the diffusion coefficients

determined from the sedimentation and diffusion studies independently. By the approach to equilibrium run an average molecular weight of 12,200 was found. This value is nearly the same as that found for a similar preparation isolated by *Yoshida et al* (18).

A small peptide, not attached to the precipitinogens, would influence Dapp even if it did not leave the meniscus on ultracentrifugation. However, if the peptide were free it most probably would have been separated from the precipitinogens by ion-exchange chromatography and/or electrophoresis. It is, therefore, reasonable to believe that it is attached or adsorbed to the precipitinogens in the purified preparation, though some of the sensitizing antigen apparently is free in the crude extract (6). On column chromatography of a crude extract (6), a fraction showing only the sensitizing ability was obtained. Hydrolysis and paper chromatographic analysis of this fraction showed the amino acids lysine, aspartic acid (trace), serine, glycine, glutamic acid and alanine (weak). Of the amino acids released by carboxypeptidase, valine and leucine thus seem to belong to the precipitinogens, whereas it is uncertain whether or not the aspartic acid and all of the alanine derive from the sensitizing component. Table 2 shows that except for alanine, only one amino acid residue of those derived from the sensitizing principle is released per molecule (Mw 12,000) by carboxypeptidase. If complete digestion with this enzyme were obtained, the sensitizing antigen should be a hexa- or hepta-peptide attached to the precipitinogens, constituting only 5 to 6 per cent of total weight of protein A.

The linkage between precipitating and sensitizing antigens is obscure. Since carboxypeptidase is active, a linkage affected through the C-terminal amino acid of the sensitizing antigen is excluded. The linkage is apparently labile. As with polysaccharide A (15), tanned sheep erythrocytes absorb all sensitizing antigen from a protein A solution whereas the precipitating ability is unaffected. The adsorption to tanned sheep erythrocytes is probably of electrostatic nature.

The number of free amino groups in each molecule (Mw 12 000) amounts to 16. This is compatible with 15 ε-amino groups of lysine and one terminal α-amino group of the chain. Previous experiments (4) showed small amounts of ε-DNP-lysine, and the only N-terminal amino acid observed was alanine. An increase in the number of free amino groups corresponding to 15 per molecule was observed after tryptic digestion. This observation agrees completely with the calculated number of lysine residues per molecule and all the ε-amino groups of lysine are apparently free. The small amount of ε-DNP-lysine observed must be due to failure in the isolation procedure rather than to blocking of the ε-amino group by a substitute as previously suggested (4).

Treatment of immune serum with mercaptoethanol at neutral pH results in conversion of the macroglobulins into smaller molecular entities (1) with loss of the antibody activity due to these macroglobulins, while the γG globulins are not affected. No differentiation of the

precipitins against protein A was obtained by mercaptoethanol treatment of sera treated and untreated sera giving the same lines and the same ring test titres. The precipitins thus seem to be of the 7S type. In the indirect haemagglutination test the titres were reduced, indicating antibodies against the sensitizing substance of 7S γ-globulins as well as of 19S γ-globulins.

Our protein A preparation seems to be composed of two closely related molecular entities, i.e. two open polypeptide chains, each having a specific precipitating ability, and small side-chains with the ability to sensitize tanned sheep erythrocytes. The side chains are probably hexa- or hepta-peptides, whereas the precipitating entities each contains about 120 amino acid residues. The main chains as well as the side-chains are digested by trypsin and all the serological activities are destroyed. Carboxypeptidase destroys the sensitizing ability only, indicating either that proline is near the C-terminal of the precipitinogens or that attack by this enzyme is sterically hindered. Since some free sensitizing principle has been found in crude extract (6) it is possible that not all precipitinogens contain side chains. If so, this could give rise to the observed heterogeneity.

It is tempting to assume that the serological activities of protein A are due to certain amino acid sequences. Different extraction methods may give preparations with various molecular size exhibiting the same serological activities.

Yoshida et al. (18) prepared their antigen by dornase digestion of undigested cell wall and observed one line only on agar diffusion against immune serum. This line corresponded to the line produced by normal human serum. The molecular weight of their preparation agrees, however, very well with that found in the present study. *Löfkvist & Sjöquist* (12) have recently extracted bacteria in the cold and isolated their protein A antigen from the supernatant after acid precipitation. This preparation (fraction A 1) showed the same serological properties as our protein A (10), whereas the sedimentation constant reported ($s^{\circ}_{20,w}$ = 2.6) (12) differs greatly from that found in the present experiments as well as from that reported by *Yoshida et al.* (18).

Boiling is a drastic way of extracting antigenic material but compared to the cold water extracted material all the serological activities are retained. As the main interest is to study the primary protein structure, it is an advantage to work with the simplest units showing the serological activities. Extraction of protein A by boiling is preferred because this procedure most probably gives simpler units than extraction by cold water.

SUMMARY

The homogeneity, molecular size and shape of protein A have been studied.

No separation of the serological activities was observed on electrophoresis

Ultracentrifugation experiments showed a small molecular heterogeneity and an average molecular weight of about 12 000 was found

Carboxypeptidase digestion destroys the sensitizing but not the precipitating activity Quantitative estimation of the amino acids released by carboxypeptidase and compared to those found in a column fraction having sensitizing but not precipitating ability shows that the sensitizing component constitutes a small part of the preparation (5 to 6 per cent) Most likely it is a hexa or hepta peptide linked as a side chain to the precipitinogens which are open polypeptide chains of about 120 amino acid residues

The observed heterogeneity on ultracentrifugation may be due to the absence of the sensitizing factor in some of the precipitating molecules

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Acta path. et microbiol. scandinav. 69: 576–594, 1967

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M. XENOPEI

A Bacteriological Study of M. xenopei including Case Reports of Danish Patients

By

H. C. ENGBÆK, B. VERGMANN, I. BAESS and D. W. WILL

Received [illegible] xi 66

Mycobacterium xenopei, a newly described pathogen, has been recognized in specimens from four Danish patients. Mycobacteria other than tubercle bacilli are isolated frequently from sputum in Denmark; in 1960–61 constituting about 10 per cent of all mycobacteria identified. Only a small number of such bacteria appear to have been pathogenic for the patients concerned; these include *M. avium*, the closely related Battey group, *M. scrofulaceum* and *M. kansasii* (6, 7, 10, 9).

First described by *Schwabacher* in 1959, *M. xenopei* was recognized to be probably pathogenic for man by *Marks & Schwabacher* in 1965. Furthermore, strains of this species have been identified by *Manten* in Holland (1965) and *Boisvert* in France (1965). A few of these strains were isolated from resected pathological lung tissue after having been found in the same patient's sputum. Though the relationship to tuberculosis-like disease is less thoroughly established, many of the isolates have been considered to be of clinical significance. The appearance of the species in urine has also been reported.

The present study includes case reports of four Danish patients and a bacteriological study of the four Danish strains together with eight received from England. The three from *Marks* were classified by him as *M. xenopei*. The five from *Nassau* were part of a number of strains sent to *Magnusson* and were supplied by the latter for use in the present study because they formed a homogeneous group in his experiments.

A. CASE HISTORIES

The following case reports are based on information provided by the hospitals and dispensaries where the patients were diagnosed and have

Thanks are [illegible] Dr. [illegible] Magnusson, Tuberculin Department of Statens Seruminstitut, for supply [illegible] sensitin preparations.

The writers are grateful [illegible] K. Tornøe and Dr. K. S. Rasmussen, Bispebjerg Hospital, Dr. K. S. [illegible] kebjerg Hospital, Dr. H. Starklint, County and Municipal Hospital, N[illegible], and Dr. C. [illegible], County and Municipal Hospital, Skive, for [illegible] to use the case histories.

been treated Comparative intradermal tests (Mantoux) were performed in March 1966 on two of the patients (Nos. 3 and 4) with sensitins produced from the following mycobacteria *M. tuberculosis* (RT 23) *M. avium* (RS 10) *Battey organism* (RS 23) *M. scrofulaceum* (RS 95) and *M. xenopei* (RS 631) The doses were RT 23 0 02 μg/0 1 ml = 1 TU other preparations 0 1 μg/0 1 ml = 5 TU The antigens were applied simultaneously on both forearms the reactions read after 72 hours and registered as the diameter of induration in millimetres

Case No. 3 F. H., male born 1903 former porcelain worker The patient was employed previously at a porcelain factory and has a history of silicosis dating from 1947 when he was hospitalized for the first time Since 1947 he has been under constant observation and treatment for that disease Roentgenograms of the lungs showed large infiltrations and cavities The patient is severely handicapped by his disease and has been in hospital repeatedly No BCG vaccination Mantoux test 1962) 1 TU human tuberculin 18 mm (72 hours)

Sputum Numerous examinations by culture since 1948 No isolation of tubercle bacilli but since 1956 *M. xenopei* isolated repeatedly with numerous to innumerable colonies As regards the bacteriological examinations see below (SSC 86)

Diagnosis Pneumoconiosis pulm (silicosis)

Treatment No chemotherapy against the mycobacteria but treatment with penicillin and sulphonamides at intervals

Case No. 4 N. male born 1895 married clerical worker formerly blacksmith Included in routine screening at the tuberculosis dispensary up to 1957 with normal findings Vaccinated with BCG On the whole well until December 1960 when it was found that he was suffering from a malignant tumour in the rectum Lab.ex.amput.recti abd.m.perineal performed with uncomplicated course During hospitalization a cavity in the upper lobe of the right lung corresponding to 1st space I II was discovered Spontaneous pneumothorax on the right side disappeared completely after treatment Three gastric lavage specimens gave no growth of tubercle bacilli and three sputum specimens were negative by microscopy Discharged from hospital in February 1961 and followed up at the tuberculosis dispensary Repeated roentgenological examinations there showed decrease in the infiltration and cysts and bronchiectasis in the right lung By April 1963 a large cavernous infiltration had developed in the middle area of the left lung Symptoms during the previous few months were fatigue and increasing dyspnoea and the temperature was probably elevated The patient was admitted to the Tuberculosis Department of Frederiksberg Hospital from April 30 to June 15 1963 SR 20 mm per hour Haemoglobin percentage 107 Mantoux test 1 TU human tuberculin 8 mm (72 hours On admission to hospital his general condition was good but he was thin and had functional dyspnoea After discharge from hospital out patient treatment and follow up

Sputum Microscopy consistently negative No isolation of tubercle bacilli but since January 1962 *M. xenopei* demonstrated by culture in six sputum specimens with few to innumerable colonies Last examination September 1964 As regards the bacteriological examinations see below (SSC 780)

Diagnosis Pneumonia (atypical mycobacteria) Tbc pulm Neopl malign recti operat

Treatment SM + PAS + INH from May 3 to August 8 1963 Total dosage SM 33 g PAS 1278 g INH 28 2 g SM + PAS + INH from April 29 to September 11 1964 T [illegible]

[illegible]

upper lobe there were large multilocular cysts in both lungs His general condition

was variable but, on the whole poor. The patient did not wish to be admitted to hospital and could not tolerate chemotherapy. He died in his home in March 1965.

Case No 3. M. male born 1919 married caretaker. Previously healthy. Never employed in any occupation likely to cause chronic lung processes. Vaccinated with BCG in 1947. In 1952 took part in routine screening at the tuberculosis dispensary. Two months before admission to hospital increasing fatigue, poor appetite and loss of weight, pain in the right side of the chest, increasing cough and expectoration, temperature about 38°. Referred to the tuberculosis dispensary because of these symptoms. Roentgenogram showed right-sided dense infiltration in i.c. space I-III. Admitted to the Tuberculosis Department of Bispebjerg Hospital in October 1963. On admission the temperature was slightly elevated during the first few days but then became normal. Discharged in May 1964. Subsequently out-patient treatment. Roentgenogram and tomogram showed a large infiltration through the upper part of the right lung from front to back. No definite cavities. Remainder of the right lung and left lung normal. SR 56 mm per hour. Haemoglobin percentage 115. Blood pressure 150/90. Urine normal. Mantoux test 1 TU human tuberculin 10 mm (72 hours).

Sputum. Microscopy revealed acid fast rods resembling tubercle bacilli. Culture during the first six weeks gave growth four times of the same kind of mycobacteria (*M. xenopei*) with moderate, few, numerous and few colonies respectively. Subsequently culture was consistently negative. Tubercle bacilli never recovered. As regards the bacteriological examination see below (SSI 989).

Diagnosis. Infilt. pulm. dxt. Cystis pulm. dxt.

Treatment. SM from November 1 1963 to May 1 1964. Total dosage 81 g.
PAS from November 1 1963. Still being treated 1966.
INH from November 1 1963. Still being treated 1966.

The lung processes decreased quite quickly during treatment and became less dense but there were still infiltrations in the upper part of the right apex when examined in September 1965. The patient was allowed to work full time from August 1964 but reports for examination every month while under out-patient treatment.

Comparative intradermal tests performed in March 1966 gave the following results: 1 TU human tuberculin RT 23: 2 mm. 5 TU avian tuberculin RS 10: no reaction. 5 TU Battey sensitin RS 23: no reaction. 5 TU *M. scrofulaceum* sensitin RS 9a: no reaction. 5 TU *M. xenopei* sensitin RS 631: 20 mm.

Case No 4. L. male born 1900 married foundry worker (ceased employment 1951). Since 1949 roentgenological processes in lungs (? silicosis). Extensive calcification in hilus. Sputum examination 1963: no tubercle bacilli by culture. Admitted to County and Municipal Hospital Nykøbing Mors, in May 1963 on account of increasing tenderness and swelling of the right scrotum. A round smooth solid tumour the size of a goose egg was found corresponding to the right testis. SR 40 mm per hour. Haemoglobin percentage 88. Blood pressure 140/75. Microscopy of urine normal.

Diagnosis. Epididymitis.

Treatment. Hemicastratio dxt.

The *testis* was macroscopically normal and the tunica vaginalis enlarged. There were small foci containing greenish pus in the epididymis. Histological examination revealed epididymitis chr. subacuta m. gr. Slight suspicion of tuberculosis. Periorchitis chr. m. gr. No chemotherapy. Post-operative course uncomplicated. Two out-patient follow-up examinations a few months later showed normal recovery. No fistula formation, no adenitis. Bacteriological examination of pus from the epididymis gave growth of *M. xenopei* with numerous colonies. For further details see below (SSI 731).

Comparative intradermal tests performed in March 1966 gave the following results: 1 TU human tuberculin RT 23: no reaction. 5 TU avian tuberculin RS 10: no reaction. 5 TU Battey sensitin RS 23: no reaction. 5 TU *M. scrofulaceum* sensitin 3 mm. 5 TU *M. xenopei* sensitin RS 631: 12 mm.

A summary of the case histories is given in Table 1.

TABLE 1
M. xenopei: *Summary of Case Histories*

Case No.	Sex	Age (years)*	Disposition	Localization of disease	Source of material	Pathogenic	Treatment	Response to treatment	Outcome of disease
1	M	53	Silicosis	Lungs	Sputum	Doubtful	None		Severely handicapped
2	M	62	Malignant tumour of the rectum	Lungs	Sputum	Yes	Chemotherapy	Transient	Deceased
3	M	44	None	Lungs	Sputum	Yes	Chemotherapy	Yes	Recovered
4	M	63	None	Epididymis	Pus	Yes	Surgery	Yes	Recovered

* *Age at time of first isolation of M. xenopei*

B BACTERIOLOGICAL STUDIES

Table 2 gives a survey of the strains studied and their origin

Isolation of bacteria All specimens from the Danish patients were inoculated primarily on Lowenstein Jensen medium after treatment with 4 per cent sodium hydroxide Growth appeared on these primary cultures for the first time after five to six weeks incubation (37°) Most of the English strains were isolated from specimens treated with sodium hydroxide (*Marks* 1966 *Nassau* 1966)

TABLE 2

M xenopei *Origin of Cultures*

Origin	Original designation	Source	SSC No
Denmark	Case 1	Sputum	786
	Case 2	Sputum	789
	Case 3	Sputum	989
	Case 4	Pus (epididymis)	791
England	Nassau TB 24	Sputum	991
	Nassau TB 38	Urine	982
	Nassau TB 40	Sputum	983
	Nassau TB 65	Gastric lavage	984
	Nassau TB 82	Urine	985
England	Marks 9889	Sputum	986
	Marks 13806	Sputum	987
	Marks 31422	Sputum	988

* SSC = Statens Seruminstitut Collection

Standard cultures All the strains were grown in Dubos fluid medium containing Tween 80 and adjusted in the Klett Summerson photoelectric colorimeter to 55 60 corresponding to about 1 mg semi dried bacilli per ml The number of bacterial units in these suspensions was determined to be about 10 per ml by colony counts on Lowenstein Jensen medium All the experiments described subsequently were performed with the standard culture or subcultures from it

Colony morphology and pigmentation Well isolated colonies of different strains on Lowenstein-Jensen medium resembled each other closely After about three weeks growth at 37° the colonies were dysgonic dome shaped without any marginal zone smooth and with yellow to pale [illegible] range pigmentation However SSC 988 showed more berry shaped colonies with slightly rough surface Only one kind of colony was found on that medium and on Dubos oleic acid albumin agar without penicillin

The *effect of light* [illegible] and pigmentation was studied both as suggested by *Runyon* [illegible] by exposure to daylight (from first growth) at room temperat[illegible] the cultures were eight weeks old

There was no change in pigmentation after short or long exposure to light. The colonies on Löwenstein-Jensen medium were butyrous and moist and emulsified easily into a finely dispersed suspension. Stained bacteria were not decolorized by alcohol and acid and were long and slender.

TABLE 3

M. xenopei *Drug Resistance*

(Figures indicate highest concentration in γ/ml of drug permitting quantitatively similar growth as in control tube. Medium: Löwenstein-Jensen)

SSC No	SM	PAS	Thiosemicarbazone (TSC)	INH	Viomycin	Cycloserine	Pyrazinamide	Ethionamide (1314)
786	2	1.6	≥ 25.6	0.05	c	c	c	c
780	c	0.4	≥ 25.6	0.05	c	c	1000	32
989	c	0.4	≥ 25.6	0.05	c	c	1000	c
791	2	0.4	≥ 25.6	0.05	c	c	1000	
991	2	0.4	≥ 25.6	0.05	c	16	c	c
932	c	c	≥ 25.6	0.05	c	c	c	c
933	2	1.6	≥ 25.6	0.32	c	16	c	c
934	c	0.4	≥ 25.6	0.08	c	c	100	c
985	c	0.4	≥ 25.6	0.05	c	c	200	c
936	c	c	≥ 25.6	c	c	c	c	c
937	2	0.4	≥ 25.6	0.05	c	c	100	32
938	c	0.4	≥ 25.6	0.05	c	c	1000	c
Control strain (No 5)	1	0.1	0.1	0.02	10	8	10	8 16

c — growth as with control strain (*M. tuberculosis*) — not examined

Resistance determinations were made on Löwenstein-Jensen medium to which the different drugs were incorporated before coagulation. The tests on Löwenstein-Jensen medium were performed with standard culture diluted 100 times, each tube inoculated with 0.1 ml. The tubes were placed horizontally until the next day when they were sealed with paraffin and incubated in vertical position at 37°. The results were read and recorded after three weeks. A highly sensitive culture of *M. tuberculosis* (Strain No. 5) was used as the control strain. The figures in Table 3 show the highest concentration of the various drugs at which the bacteria were able to grow to the same extent as on the control tubes, i.e. with 100 or more colonies per tube. Almost all the strains of *M. xenopei* were sensitive to streptomycin, viomycin, cycloserine and ethionamid (1314). The degree of resistance to PAS and INH varied slightly but the values were never high. There were large variations from strain to strain with pyrazinamide. Repeated experiments with the same strains indicate that these differences were due to technical difficulties encountered when working with that drug. In these cases the

highest value is given in Table 3. Only to thiosemicarbazone (TSC) were all the strains strongly resistant. Furfuroyl-2-hydrazine (not shown in the table) had no influence on the growth as tested at 10, 20 and 40 µg/ml (*Bönicke* 1958). 100 units of penicillin per ml in Dubos oleic-acid albumin agar caused strong inhibition of all strains, although with four of the strains a small percentage of the bacterial units were able to grow.

The *effect of temperature* was determined by examining tubes of Löwenstein-Jensen medium inoculated with 0.1 ml of dilutions of the standard culture incubated at 22°, 37°, 40°, 43° and 45°. Readings and recording were made after 1, 2, 3, 4 and 8 weeks. The results in Table 4 refer to the first time (in weeks) at which well isolated colonies were visible. No growth after eight weeks is recorded as 0. All the strains grew at 45° but not at 22°, even after an observation period of eight weeks. At temperatures of from 37° to 45° there was no systematic difference in the time at which growth was first observed. Results not given in the table show that this also applied both as regards the maximum number and the size of the colonies.

TABLE 4

M. xenopei. *Effect of Temperature on Growth on Löwenstein-Jensen Medium*

SSC No.	First visible growth (weeks)* 22°	37°	40°	43°	45°
786	0	2	2		2
780	0	2	2		2
989	0	2	2	2	2
791	0	2	2	2	2
981	0	3	8	3	3
982	0	3	3	3	3
983	0	4	4	4	4
984	0	3	3	2	3
985	0	3	3	2	2
986	0	4	4	4	4
987	0	3	3	3	3
988	0	3	3	3	3

0 = no growth after eight weeks
— = not examined

* See footnote p. 591

C. BIOCHEMICAL TESTS

Methods

Amylase test (*Bönicke* 1960). About 100 mg of culture was scraped from Löwenstein-Jensen slants and transferred into a previously weighed tube. The bacterial mass was comminuted with a glass rod and sterile saline added gradually. The bacteria were then washed twice with 10-15 ml of sterile saline and emulsified in phosphate buffer (pH 7.2) to a concentration corresponding to 10 mg per ml. Five tenths ml of each amide solution (0.001 M) was placed in a centrifuge tube and 0.5 ml of the bacterial suspension added. Simultaneously one drop of culture was inoculated on a blood agar plate as control for contamination. The

centrifuge tubes were placed in the incubator at 37° for about 19 hours. The test was then performed by adding manganese sulphate, phenol and hypochlorite as described by *Bönicke*. After boiling for 15 minutes the tubes were centrifuged at 3000 r.p.m. for 15 minutes and the colour of the supernatant measured in the Coleman Junior spectrophotometer at a wave length of 5.5 mμ. The direct concentration of ammonia was determined by reference to a standard ammonia curve. The degrees of reaction (γ per ml) were recorded as follows: 0 – <2; ± – >2 to 5; ++ – >5 to 10; +++ – >10. The amides are numbered as suggested by *Bönicke*, as follows: 1 Acetamide, 2 Benzamide, 3 Carbamide, 4 Isonicotinamide, 5 Nicotinamide, 6 Pyrazinamide, 7 Salicylamide, 8 Allantoin, 9 Succinamide, 10 Malonamide.

Phenolphthalein sulphatase test (*Wayne* modification 1962b). 10 ml of Dubos fluid medium with Tween 80 in a Coleman spectrophotometer cuvette was inoculated with 0.1 ml of a tenfold dilution of standard culture and incubated at 37°. Suspension of the bacteria was facilitated by using a 15 mm long plastic sealed magnetic bar in the cuvette. The growth was measured at intervals of a few days until an optical density of 0.100 at 580 mμ was obtained. A sterile solution of potassium phenolphthalein disulphate was then added to a final concentration of 0.001 M. Incubation was repeated at 37°. Samples of 0.5 ml were taken after 3 days and after 1, 2, 3 and 4 weeks and equal parts of a 1 M sodium carbonate solution added. The hydrolysis of the phenolphthalein was evaluated by means of the resulting pink colour and recorded after 30 minutes in five grades from (–) to ++++.

Semi-quantitative catalase test (*Wayne* 1962a). Löwenstein-Jensen medium was coagulated in test tubes placed in upright position. The surface was inoculated with 0.1 ml of a tenfold dilution of standard culture. An aluminium cap was placed loosely on the tube, which was then incubated at 37° for three weeks. 1 ml of a mixture of equal parts of 10 per cent Tween 80 and 30 per cent hydrogen peroxide was added. When foaming reached its maximum, the height of the foam column was measured in millimetres and recorded as follows: ± – from few bubbles to 5 mm; ++ – >5 to 30; +++ – >30 to 60; ++++ – >60.

Niacin test. Since large, fully grown colonies are the most suitable for this test, four to five week old cultures on Löwenstein-Jensen medium were used. In order to promote growth the tubes had been closed for two to three weeks with loosely fitting aluminium caps. About 2 ml of sterile 0.9 per cent sodium chloride solution was added and the tube placed in boiling water for one hour. After cooling a 1 ml sample was taken and the Konno test performed by adding 1 ml of 4 per cent aqueous potassium cyanide solution and immediately afterwards 1 ml of 5 per cent chloramine solution. If the mixture was opaque it was centrifuged before the reaction was read. After five minutes the yellow colour was measured in the Coleman spectrophotometer at 400 mμ. The amount of nicotinic acid was determined by reference to a standard nicotinic acid curve.

Tween 80 degradation test (*Wayne* modification 1962a). One loopful of a three to four week old culture was taken from Löwenstein-Jensen medium and emulsified in 4 ml 1/15 M phosphate buffer pH 7.0 with 0.5 per cent Tween 80. Neutral red was added as indicator. The bacterial suspension was incubated at 37°. Degradation by Tween causes the fluid to become red as acid is liberated. Observation for change of colour was performed after four hours. If there was no reaction at that time the observation was repeated once a day for seven days.

Nitrate reduction test (*Wayne* modification 1962b). The bacteria were grown in a Coleman spectrophotometer cuvette (as described under the phenolphthalein sulphatase test) in 10 ml of Dubos fluid medium with Tween 80 containing 0.01 M sodium nitrate. When the required optical density was reached an aliquot of 0.1 ml was taken and added to 8.5 ml of a 0.006 per cent solution of sulphanilamide in 0.1 N hydrochloric acid. 1.5 ml of 0.02 per cent N-(1-naphthyl) ethylene diamine dihydrochloride solution was then added and the optical density measured after 5 min at 530 mμ. The concentration of nitrite in the sample was determined by reference to a standard nitrite curve.

Results

Details of the experimental results are shown in Table 5. It is of special interest that the reactions to amides 5 (nicotinamide) and 6

TABLE 5
M xenopei *Biochemical Tests*

NSC N	Amylase reaction 5*	6†	Others	Phenophthalein sulphatase Reaction	Day	Catalase	Niacin γ/ml	Tween degradation Reaction	Day	Nitrate reduction γ/ml
56	+	++	0	++	3	+	0 9	0	7	20
780	+	++	0	+++	3	+	0	0	7	10
189	+	+	0	+++	3	+	1 1	0	7	10
791	+	+	0	+++	3	++	1 8	0	7	10
991	+	+	0	+++	3	+	0 3	0	7	0
992	+	+	0	++	3	+	0 7	0	7	0
993	+	++	0	++	3	+	1 6	0	7	0
994	+	++	0	++	3	+	0 7	0	7	0
995	+	++	0	++	3	+	0 7	0	7	0
986	+	++	0	++	3	+	1 5	0	7	0
987	+	++	0	+++	3	+	1 4	0	7	0
988	+	++	0	+++	3	+	2 2	0	7	0

* 5 — Nicotinamide † 6 — Pyrazinamide

(pyrazinamide) were positive that the sulphatase reaction occurred rapidly and was generally strongly positive and that the catalase reaction was only weak

D PATHOGENICITY AND VIRULENCE

Methods

Experimental animals *Hens* (weight 2100 2400 g) and *rabbits* (weight 1900 2100 g) infection dose 5 mg intravenously—in addition with one of the strains (SSC 91) 1 and 10 2 mg intravenously observation time 3 months The hens showed no reaction to avian tuberculin before infection *Guinea pigs* (weight 275 375 g) infection doses 1 mg intravenously 1 mg intraperitoneally 0 1 mg intracutaneously in two places observation time 2 months *Mice* (weight 18 20 g) Albin strain (C) black mice (C57) and red mice (Clethrionomys glareolus Schreb) infection dose 10 mg intraperitoneally observation time 2 months

Recording Macroscopical changes in the visceral organs of all animals and in rabbits any processes occurring in the joints and tendon sheaths of the legs were recorded as 0 + + + The local reactions in guinea pigs after intracutaneous injection were measured and evaluated after 1 2 3 4 and 8 weeks *Smears* were made from liver spleen and lungs of all the mice and some of the other animals and stained by the Ziehl Neelsen method with 3 4 per cent aqueous picric acid as contrast stain The numbers of bacteria were recorded as + 1 50 ++ 51 300 +++ 2 70 per individual oil immersion sight field ∞ innumerable Observation time four minutes In the mouse experiments the number of bacteria in the smears are expressed in terms of the following index —

0 — no bacteria
1 — 1 50 bacteria
2 — 51 300 bacteria
3 — 2 per sight field to ∞ bacteria

The figures in Table 11 show the distribution of the twelve strains of *M xenopei* according to the average index size The average index is worked out per organ per mouse and shown separately for the three mouse breeds

Culture was performed on tissue from all the animals except the mice Before inoculation the material was comminuted in mortar and treated with 4 per cent NaOH Inoculation was made on four tubes of Löwenstein-Jensen medium which were incubated at 37° The results of culture were registered as follows according to the number of colonies per tube + 1 10 ++ 11 49 +++ 50 99 ∞ ≥ 100

Results

Hens The strains were inoculated into 16 hens It will be seen from Table 6 that 12 of the animals died in the course of the observation period the first on the 35th and the last on the 75th day Eight animals had macroscopically visible lesions in spleen (mainly enlargement) seven in liver and one in lungs Culture revealed large numbers of bacteria in one or more organs in all the animals (except one) regardless of time of death and extent of macroscopically visible lesions However microscopical examination of smears showed relatively small numbers of bacteria

Titration of the virulence with one of the strains (SSC 791) is shown in Table 7 The survival times were dependent on dosage With 10 mg all three hens survived the whole observation period With 5 and 1 mg the spleen was enlarged in all animals except one and innumerable

TABLE 6

M. xenopei Virulence Determination – Hens (5 mg i.v.)

SSC No.	Day of death or killed (3 mths)	Spleen Culture No. of colonies	Spleen Microscopy Acid fast bacilli	Liver Culture No. of colonies	Liver Microscopy Acid fast bacilli	Kidney Culture No. of colonies	Lung Culture No. of colonies	Lung Microscopy Acid fast bacilli
786	39	∞		∞			+++	
790	42	∞		∞			+++	
59	51	∞		∞			∞	
791	35	∞	+	∞	∞	++	∞	0
	37	∞		∞			cont	
991	k	+	0	∞	0		∞	0
992	k	+++	0	++	+	0	cont	0
993	k	∞	+	∞	+	∞	∞	+
	k	0	0	+	0	0	0	0
984	64	∞	+	∞	++	+	+++	0
995	75	∞	+	∞	0	∞	∞	+
996	k	+	0	∞	0	+	+	0
	56	∞	0	cont	0	+	++	0
997	78	∞	0	∞	0		cont	0
	56	∞	++	∞	0	++	∞	0
998	46	∞	∞	∞	++	++	++	0

cont — contaminated   — not examined

colonies were recovered from one or more organs in all cases. With 10^{-*} mg there were no macroscopically visible processes and only one of the hens had large numbers of bacteria in the organs.

Rabbits. The strains were examined in 13 rabbits. It will be seen from Table 8 that all the rabbits except two survived the observation period. The rabbit which survived only 23 days presumably died of

TABLE 7

M. xenopei. *Titration of Virulence in Hens with SSC 791*

Dose i.v.	Day of death or killed (3 mths)	No. of colonies by culture		
		Spleen	Liver	Lung
5 mg	22	∞	∞	∞
	33	∞	∞	cont
	28	∞	∞	∞
	33	∞	∞	∞
	k	∞	+	+
1 mg	41	∞	∞	∞
	54	∞	∞	∞
	40	∞	∞	∞
	27	∞	∞	+++
	47	∞	+++	∞
10^{-*} mg	k	0	0	0
	k	0	0	0
	k	∞	∞	+

The table includes the results of four separate experiments.
cont. = contaminated

TABLE 8

M. xenopei. *Virulence Determination. Rabbits* (5 mg i.v.)

SSC No.	Day of death or killed (3 mths)	No. of colonies by culture				
		Spleen	Liver	Kidney	Lung	Joint
786	66	+	++		+	0
780	k	0	∞		0	0
989	k	+	+	0	+	0
791	23	+++	++		+++	0
	k	+	++	+	++	0
991	k	+	+	0	+	0
982	k	0	+	0	+	+
983	k	+	++	+	+	0
984	k	0	+	+	+	0
985	k	0	+	0	+	0
986	k	+	++	0	+	0
987	k	+	+	0	0	+
988	k	+	+	0	+	0

— = not examined

intercurrent disease, since six other rabbits infected with the same strain all survived the infection (Tables 8 and 9) There were only few macroscopically visible lesions in the organs and the joints and tendon sheaths were not affected Culture gave growth of far less colonies than in the above mentioned experiment on hens From only two animals could a few colonies be isolated from joints

Titration of virulence for rabbits was also undertaken with strain SSC 791 The results of culture (Table 9) show that the number of bacteria recovered from the organs was far less than in the hen experiment

TABLE 9

M xenopei *Titration of Virulence in Rabbits with SSC 791*

Dose i v	Day of death or killed (3 mths)	No of colonies by culture Spleen	Liver	Lung	Joint
5 mg	k	0	++	0	+
	k	0	+	0	0
	k	0	+	+	+
	k	+	+++	+	0
	k	0	++	+	0
1 mg	k	0	+	0	0
	k	0	+	0	0
	k	0	+	0	+
	k	0	+	0	0
	k	0	+	0	+
10^{-2} mg	k	0	0	0	0
	k	0	0	0	0
	k	0	+	0	0

The table includes the results of four separate experiments

Guinea pigs The strains were injected intraperitoneally into 25 animals The macroscopically visible lesions were limited to the site of injection in the form of necrotic nodules in the omentum in about 50 per cent of the animals Small numbers of bacteria could be recovered from the omentum as well as from one or more organs

A similar number of animals were injected intravenously All survived the observation period and only few bacteria could be recovered from one or more organs

Intracutaneous injection of the bacteria (Table 10) produced erythematous nodules of varying size with maximum ulceration after about four weeks This could still be seen after eight weeks with five of the strains though generally by then the size of the lesions had decreased considerably and one had healed completely

Mice The examinations with the three breeds of mice showed that only a small number of animals died after the intraperitoneal injection of 10 mg The deaths occurred particularly among the house and red

mice, but the findings were not reproducible in repeated experiments with the individual bacterial strains. Autopsy after two months showed practically no macroscopically visible lesions in the organs

It will be seen from the distributions given in Table 11 that the three breeds of mouse reacted differently to infection with *M xenopei* The largest number of bacteria were found in the house mice and the smallest number in the red mice This applies to all three organs examined Furthermore, the index for the individual breeds indicates that the number of bacteria was greatest in the spleen and lowest in the lungs This was confirmed by comparing the results in the individual animals

TABLE 10

M xenopei *Size of Local Reactions (mm) after Intracutaneous Injection of 0 1 mg into Guinea Pigs*

SSC No	weeks 1	2	3	4	8
786	5	14	–	12*	12*
780	5	5	8	8*	3
939	8	11*	15*	14*	9*
791	5	7	10*	11*	6
991	5	6	7	7*	5
982	3	6	8	7	7
983	8	9	7	8*	6*
984	6	7	8*	6*	4*
985	6	7	12*	8*	4*
986	7	7	3	8*	2
987	7	15	16	12*	0
988	8	8	11*	8*	2

* = ulceration   – = not examined

TABLE 11

M xenopei *Mice Experiments*
Distribution of 12 Strains According to Average Bacterial Index Calculated per Mouse per Organ (See Text)

Average index	House mice Spleen	Liver	Lung	White mice Spleen	Liver	Lung	Red mice Spleen	Liver	Lung
0	0	0	2	0	2	4	1	4	7
0 1 – 1 4	5	7	10	9	9	8	10	8	5
1 5 – 2 4	4	3	0	3	1	0	1	0	0
2 5 3 0	3	2	0	0	0	0	0	0	0

Only the mice which survived the two months observation period are included in the evaluation

Index 0 = no bacteria
1 = 1–50 bacteria
2 = 51 300 bacteria
3 = 2 *per sight field to ∞ bacteria*

F. HISTOPATHOLOGY

In connection with some of the autopsies, pieces of tissue were removed from liver, spleen, lung and kidney, and placed in 10 per cent neutral formalin solution for histopathological examination. Haematoxylin eosin and acid fast stained sections were examined.

TABLE 12

M. xenopei. Histopathological Findings

SSC No.	Day of death or killed (3 mths)	Spleen	Liver	Kidney	Lung	Acid fast bacilli
Hens						
791	35	++++	++++	++	++	Innumerable
982	k	+	++	0	+	Rare
983	k	++	++++	0	++	Innumerable
	k	0	0	0	0	None
984	64	++++	++++	++	+	Innumerable
985	75	++++	++++	0	+	Innumerable
986	k	++++	+++	0	++	Innumerable
	56	+++	++++	+	+	Innumerable
987	38	+++	+++	*	0	Innumerable
	56	++++	++++	+	+	Innumerable
988	46	++++	++++	0	+	Innumerable
Rabbits						
791	k	*	++	0	0	None
991	k	0	0	0	0	None
Guinea Pigs						
791	k	0	+	0	0	None
991	k	+	+	0	0	None
	k	0	+	0	0	None

++++ — Innumerable granulomata with confluence
+++ — Many discrete granulomata
++ — Scattered discrete granulomata
+ — Rare typical granulomata
0 — No recognizable granulomata
* — No tissue

The essential histopathological lesion in the hen was a small non-caseating granuloma composed of irregular large epithelioid cells with pale cytoplasm surrounded by a thin rim of lymphocytes and reticulum cells. In heavier infections the granulomata were confluent but retained at least a degree of morphological integrity. No necrosis of any type was observed in the granulomatous lesions though surrounding parenchymal tissues, particularly the liver, might show extensive coagulative necrosis when innumerable granulomata were present. In those parenchymal organs sampled (liver, spleen, lung and kidney) no specific patterns of distribution of the mycobacterial lesions were observed.

Acid fast bacilli were present in the granulomata in almost all

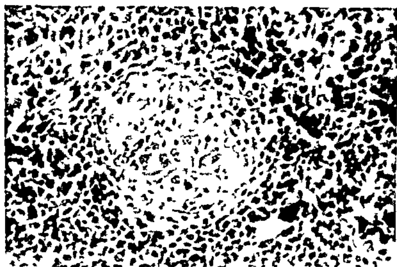


Fig 1
M xenopei (SSC 986) Granulomatous lesions in spleen of hen killed after three months (haematoxylin eosin stain magnification 420 times)

instances in large numbers and were not seen in other areas The organisms were long usually beaded and strongly acid fast The small but typical granulomatous lesions seen in the liver of rabbits and guinea pigs contained no organisms and are assumed to represent abortive or healing granulomata induced by mycobacterial lipoprotein

The results of the histopathological examinations are shown in Table 12 and Fig 1

DISCUSSION

In the present work the four Danish strains form a homogeneous group indistinguishable from the English strains three of which have been classified by *Marks* as *M xenopei*

The bacteriological findings generally confirm what has been reported from other sources (15 16 1 13) The bacteria grow slowly with dysgonic colonies of pale to moderate orange colour There is no growth at 22 but good growth at 45° and the bacteria are sensitive or relatively sensitive to a number of primary and secondary drugs

Other results of particular interest are the positive reactions to amides 5 (nicotinamide) and 6 (pyrazinamide) i e a pattern said to be characteristic for *M avium* and strains resembling *M avium* (*Bonicke* 1960) Furthermore there is moderate to strong positive sulphatase reaction on the third day a result which has hitherto been recorded only with rapidly growing strains (*M fortuitum*) (12 21 20) The animal experiments reveal a characteristic difference in the ability of *M xenopei* to multiply in hens and rabbits both as regards survival time

and recovery of bacteria from the organs The pathogenicity of the strains for hens has been emphasized recently by *Boisvert* (1965)

Comparison with *M avium* is of importance because of a number of mutual characteristics which might give rise to incorrect diagnosis i e colony morphology pigmentation rate of growth good growth at 43° weak catalase reaction, and amide pattern However *M xenopei* is distinguishable from *M avium* in a number of ways lack of growth at 22° (with *M avium* almost all strains grow in the course of eight weeks) good growth of all strains at 45° (with *M avium* about 70 per cent of the strains give growth and of these only about one-fifth give growth of the same number of colonies as at 37°) the rapidly occurring sulphatase reaction and the relative sensitivity to a number of drugs

As regards the pathogenicity of the strains and their virulence for experimental animals it is the experiments with hens which are of particular interest Comparison with a number of strains of *M avium* isolated from pigs shows that *M xenopei* is somewhat less virulent for hens than *M avium* even though a dose of 1 5 mg injected intravenously caused multiplication of the bacteria in the organs and death of many of the animals The weaker virulence is manifested by the lower lethality the longer survival time and the finding by microscopy of relatively few bacteria in the organs

The histopathological findings resemble those observed with *M avium* The distribution of lesions is similar to that found with other mycobacteria of low virulence for hens e g strains of the Battey group

The difference in the virulence of *M xenopei* and *M avium* is evident when tested in rabbits and guinea pigs infected intravenously and in mice infected intraperitoneally After infection with *M xenopei* almost all the rabbits survived and it is of special interest that none of them showed signs of chronic processes in particular in joints and tendon sheaths Such processes are characteristic after infection with *M avium* and strains resembling *M avium* (4 11 10 5 8 24) The guinea pigs also survived the infection and almost all the animals both rabbits and guinea pigs had only few bacteria in their organs In contrast in infections with *M avium* almost all the rabbits die and large numbers of bacteria can be recovered from the organs About 70 per cent of the guinea pigs die spontaneously with large numbers of bacteria in one or more organs this applies furthermore to most of the surviving animals In the mouse experiments also *M xenopei* is less capable than *M avium* of persisting or multiplying in the organs This is seen with all three mouse breeds and can be demonstrated in spleen liver and lung

As regards the pathogenicity of *M xenopei* for man patient No 4 in this study is of particular interest since this is the first case in which it has been demonstrated that *M xenopei* can provoke extrapulmonary disease Other reports where information is available concerning the patients mention only pulmonary processes (1 13 16) The present

material is too small and non-homogeneous to permit the drawing of prognostic conclusions. However, the sensitivity of the strains to a number of primary and secondary drugs provides other possibilities of treatment than in infections with *M. avium* and other mycobacteria, *e.g.* strains belonging to Runyon's Group III. Patient No. 3 was apparently cured by means of chemotherapy. In Case No. 2 there may also have been a considerable benefit from treatment which, however, could not be effectuated in full because of hypersensitivity and other circumstances. Surgical removal of the local process without chemotherapy was sufficiently effective treatment in the patient with epididymitis (Case No. 4).

The results of comparative intradermal testing indicate that the sensitin produced from *M. xenopei* can be of assistance in determining whether or not a strain is pathogenic for the patient from whom it was isolated.

With a view to ascertaining the frequency of *M. xenopei*, review has been made of a material of mycobacteria other than tubercle bacilli isolated from about 350 Danish patients, including all strains which grew at 43° in subculture. It was found that only the strains isolated from the four patients in this study showed the characteristic mosaic of findings. It can be concluded, therefore, that *M. xenopei* occurs only infrequently in Denmark.

SUMMARY AND CONCLUSION

A description is given of the course of disease in the four Danish patients from whom *M. xenopei* was isolated. In three of these the strain was definitely pathogenic: two provoked lung processes and the third abscesses in the epididymis.

The bacteriological examinations included the four Danish and eight English strains. The study shows that the strains formed a homogeneous group with a characteristic mosaic of properties not observed with previously described species of mycobacteria. On account of a number of similarities with *M. avium*, a comparison with that species is included in the discussion.

M. xenopei occurs infrequently in Denmark. It has been demonstrated only in the four patients in this study out of about 350 from whom mycobacteria other than tubercle bacilli have been isolated.

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Subsequent experiments with some of the strains showed that growth at 43 45° occurred one to two weeks earlier than growth at 37 40°

Acta path. et microbiol. scandinav. 69: 593–609, 1967

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CHANGES IN CIRCULATING LYMPHOCYTE POPULATIONS IN PERTUSSIS VACCINATED GUINEA PIGS

A Quantitative Study of Blood and Thoracic Duct Lymphocytes in Normal, Sham Operated and Thymectomized Animals

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Received 9.xi.66

The importance of an intact thymus for normal morphological and functional development and regeneration of the lymphoid system in rodents is well documented (refs. see *Ernström* 1965). The role of the thymus, if any, during an immune response is not, however, known. *Fichtelius et al.* (1957, 1958) reported increased cell formation in the rat thymus after treatment with pertussis vaccine, thus indicating a contribution of the thymus to the associated lymphocytosis in the blood. *Miller* (1963) and *Metcalf* (1961) nevertheless denied the existence of mitotic stimulation in the thymus during an immune response.

In previous papers we presented evidence of an emigration of small lymphocytes from the thymus. Thus a greater number of small lymphocytes per mm³ of blood was found in the thymic veins than in the afferent arterial blood (*Ernström & Larsson* 1966 a). An increase in this venous output of small lymphocytes from the thymus could be demonstrated after administration of thyroid hormone (*Ernström & Larsson* 1966 b). The object of the present investigation was to study the cellular emigration from the thymus during an immune response and to compare the response of different categories of blood lymphocytes to antigenic stimulation in sham operated and thymectomized animals. In view of the aforementioned results of *Fichtelius et al.* indicating a possible antigen stimulated emigration of cells from the thymus we used pertussis vaccination as the experimental model.

MATERIAL AND METHODS

Totally 211 male guinea pigs with an initial weight of 237±2 g (mean ± S.E.) were used. The animals were fed on cabbage, turnips, carrots and vitamin pellets. They were divided into three main groups, i.e. non-operated, sham operated and thymectomized guinea pigs (70, 71 and 70 animals respectively). Operation was performed under local anaesthesia (subcutaneous infiltration with 0.5 per cent Xylocaine® Astra) using the technique of *Gyllensten* 1953. The animals were in

jected subcutaneously in the back with 0.5 ml of pertussis vaccine (commercial preparation from the State Bacteriological Laboratory, Stockholm, Sweden) containing 20×10^9 killed bacteria per ml. In the operated animals injection was performed immediately after sham operation or thymectomy.

In each group 10 guinea pigs were examined 1, 3, 6, 9, 12, 15 and 21 days after the vaccine injection between 9 a.m. and 3 p.m. They were anaesthetized with 2.5 per cent Nembutal sodium® (25–50 mg/kg b.w. i.p.). In the non operated and sham-operated animals the thymus was exposed and the largest of the thymic veins incised. A blood sample was taken in a pipette for supravital staining and for white cell counts.

In all animals the right carotid artery was incised near the origin of the thymic artery and samples of arterial blood were taken for analysis as above. Immediately afterwards the artery was ligated. The confluence of the thoracic duct with the left subclavian and jugular veins was then exposed, essentially according to the technique of *Reinhardt & Yoffey* (1957), although the sternum was not incised. The thoracic duct was punctured and a sample of lymph was collected in a pipette for supravital staining. The whole procedure was performed under a dissection microscope. Finally the thymic lobes were removed and weighed and the animals killed. The supravital specimens of blood and lymph were stained with Janus green B and neutral red (see *Ernström & Larsson* 1966 b), and were immediately studied under a microscope at 1 000 × magnification. Differential counts were made of lymphocytes with different mitochondrial content. The lymphocytes were registered in six classes: cells with 0–5, 6–10, 11–15, 16–20, 21–30 and >30 mitochondria. The average number of mitochondria per lymphocyte was calculated for each sample from the percentages of lymphocytes and the median number of mitochondria in the different classes. For convenience, 0–10, 11–20 and >20 mitochondria per cell are denoted as a low, medium and high mitochondrial content respectively. The mitochondrial content (MC) is correlated to the size of the lymphocytes: low MC corresponds to small lymphocytes, high MC to large lymphocytes (*Wiseman* 1931, *Fichtelius & Larsson* 1961, *Ernström & Larsson* 1963).

For determination of the absolute number of white cells 25 mm³ of blood was diluted with 475 mm³ of Türk's solution (containing methyl violet for staining of white cells). The number of cells was counted in a Bürker counting chamber 288 squares (corresponding to 0.00625 mm³ each) in each sample. A differentiation was made between mono- and polynuclear white cells.

The results were analyzed statistically by Student's *t* test. The comparisons between percentage and number of lymphocytes in thymic vein and carotid artery blood were performed by statistical analysis of the difference in individual animals. The p values <0.001, <0.01 and <0.05 are denoted as highly significant, significant and almost significant respectively.

RESULTS

Operation did not affect the growth of the animals, which was identical in all three groups. No mortality occurred. The mean thymic weight decreased slightly but not significantly after the vaccine injection being minimal 6 days afterwards (– 10 per cent).

Antigen Treated Non Operated Animals

A single injection of pertussis vaccine caused a slight increase in the number of lymphocytes in the blood 9–21 days afterwards. The number of granulocytes per mm³ of blood also increased being maximally raised 9 days after injection. The normal excess of lymphocytes in the thymic vein blood as compared to the carotid artery blood (*Ernström & Larsson* 1966 a) persisted after vaccine injection, but no significant increase or decrease in this excess could be demonstrated

TABLE 1

Comparison Between Differential Counts of Lymphocytes in Carotid Artery and Thymic Vein Blood of Guinea Pigs at Different Intervals after a Single Injection of Pertussis Vaccine Mean ± SE.

Source of blood	Days after pertussis vaccination	No of animals	Classification of lymphocytes by mitochondrial content Lymphocytes in per cent 0 5	6 10	11 15	16 20	21 30	> 30	Average no of mitochondria cell	p value of difference
Thymic vein	1	10	22±0.9	41±1.0	23±1.4	8±0.8	3±0.3	0±0	9.4±0.1	
Carotid artery	1	10	17±0.7	37±1.4	30±1.0	12±1.1	4±0.4	0.2±0.2	10.6±0.2	p < 0.001
Thymic vein	3	10	26±1.7	47±1.6	20±1.5	6±0.8	2±0.3	0±0	8.6±0.2	
Carotid artery	3	10	18±1.4	43±2.2	26±1.6	9±0.7	3±0.4	0.2±0.2	9.9±0.2	p < 0.001
Thymic vein	6	10	10±0.8	34±1.1	33±1.6	16±1.0	7±0.4	0.4±0.3	12.1±0.1	
Carotid artery	6	10	5±0.6	24±2.1	38±1.6	21±1.6	10±0.7	2±0.3	14.0±0.3	p < 0.001
Thymic vein	9	10	13±0.8	42±1.2	27±1.3	12±1.5	5±0.2	0±0	10.5±0.2	
Carotid artery	9	10	8±0.8	33±1.5	36±1.4	16±1.5	7±0.6	0±0	12.1±0.2	p < 0.001
Thymic vein	12	10	20±1.1	46±1.0	19±0.9	6±0.5	2±0.5	0±0	8.7±0.1	
Carotid artery	12	10	16±0.9	19±1.5	28±1.3	12±0.9	4±0.6	0±0	10.6±0.2	p < 0.001
Thymic vein	15	10	8±0.5	36±2.3	30±1.4	19±2.3	7±0.7	0.8±0.3	12.3±0.4	
Carotid artery	15	10	5±0.4	25±2.2	34±1.3	23±0.9	10±0.8	2±0.5	14.0±0.2	p < 0.01
Thymic vein	21	10	19±0.5	43±0.9	25±0.9	9±0.7	5±0.3	0.2±0.2	10.0±0.1	
Carotid artery	21	10	11±0.6	36±1.9	29±1.2	15±1.4	7±0.5	0.8±0.4	11.9±0.3	p < 0.001

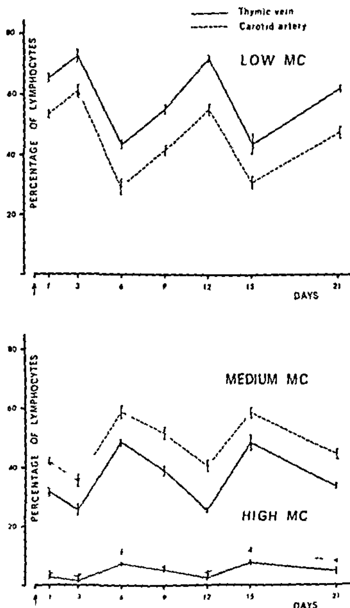


Fig 1

Percentage of lymphocytes with different mitochondrial content (MC) in thymic vein and carotid artery blood of non operated guinea pigs at different intervals after a single injection of pertussis vaccine

The lymphocytes were subdivided into classes by their mitochondrial content (Table 1). The percentage of blood lymphocytes with different mitochondrial content (MC) changed cyclically during the immunization procedure. Thus the percentage of lymphocytes with low MC (small lymphocytes) had a first maximum 3 days and a second one 12 days after pertussis injection. Concurrently, a corresponding mini

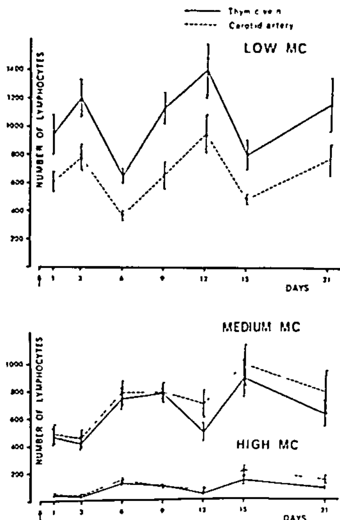


Fig 2

Number of lymphocytes with different mitochondrial content (MC) per mm³ of thymic vein and carotid artery blood of non operated guinea pigs at different intervals after a single injection of pertussis vaccine

mum was noted in the incidence of lymphocytes with medium and high MC (Fig 1)

Similarly the number of blood lymphocytes with different MC changed cyclically (Fig 2), these changes being significant The number of "small" lymphocytes per mm³ of blood in the thymic vein, as well as in the carotid artery, decreased from day 3 to day 6 ($p<0.001$, $p<0.001$) increased from day 6 to day 9 ($p<0.001$, $p<0.01$) and again decreased from day 12 to day 15 ($p<0.05$, $p<0.01$) During the whole immunization period, there was an excess of "small" lymphocytes in

TABLE 2

Comparison Between Differential Counts of Lymphocytes in Carotid Artery Blood of Sham Operated and Thymectomized Guinea Pigs at Different Intervals after a Single Injection of Pertussis Vaccine Mean ± SE

	Days after pertussis vaccination	No of animals	Classification of lymphocytes by mitochondrial content Lymphocytes in per cent 0 5	6 10	11 15	16 20	21 30	> 30	Average no of mitochondria/cell	p value of difference
Sham operated	1	10	16±0 7	41±0 7	27±1 1	12±1 0	4±0 2	0±0	10 4±0 1	p < 0 001
Thymectomized	1	10	8±0 7	32±1 9	33±0 9	19±0 9	7±0 7	0 6±0 3	12 5±0 3	
Sham operated	3	10	17±1 1	40±1 0	27±1 0	12±0 8	3±0 3	0±0	10 2±0 4	p < 0 001
Thymectomized	3	10	5±0 6	17±2 1	39±1 5	25±1 6	10±0 6	2±0 4	14 5±0 1	
Sham operated	6	10	4±0 4	18±0 5	40±1 2	26±1 2	10±0 6	2±0 6	14 6±0 1	p < 0 001
Thymectomized	6	10	2±0 4	13±0 8	37±1 7	30±1 5	12±2 9	5±0 8	16 6±0 1	
Sham operated	9	11	4±0 5	17±1 5	37±1 4	27±1 4	12±0 7	3±0 7	15 3±0 2	p < 0 001
Thymectomized	9	10	0 1±0	10±0 9	35±1 1	30±1 4	20±0 7	5±0 7	17 1±0 2	
Sham operated	12	10	18±0 8	40±0 6	28±1 0	10±0 8	4±0 2	0 4±0 4	10 3±0 2	p < 0 001
Thymectomized	12	10	3±0 4	17±1 0	40±1 7	23±1 2	15±0 9	2±0 6	15 3±0 1	
Sham operated	15	10	5±0 5	20±2 2	36±1 3	26±1 2	11±0 3	2±0 5	14 5±0 2	
Thymectomized	15	10	7±0 8	20±0 9	38±1 4	21±1 5	11±0 8	3±0 5	14 3±0 2	
Sham operated	21	10	10±0 5	32±0 9	33±1 1	17±0 7	8±0 6	0 4±0 3	12 3±0 2	p < 0 001
Thymectomized	21	10	8±0 6	20±1 0	37±1 2	23±1 0	12±0 8	1±0 3	14 0±0 1	

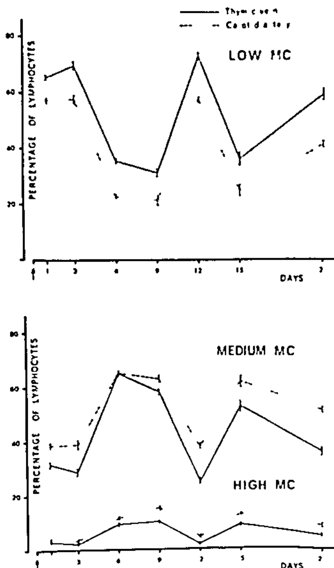


Fig 3

Percentage of lymphocytes with different mitochondrial content (MC) in thymic vein and carotid artery blood of sham operated guinea pigs at different intervals after a single injection of pertussis vaccine

the blood from the thymic vein as compared to that from the carotid artery This excess was also significant (p<0 001 p<0 001 p<0 001 p 0 001 p<0 01 p<0 01 p<0 01 respectively 1 3 6 9 12 15 and 21 days after the vaccine injection) No significant increase or decrease in this excess of small lymph cytes in the thymic vein blood demonstrable as a result of the antigen injection

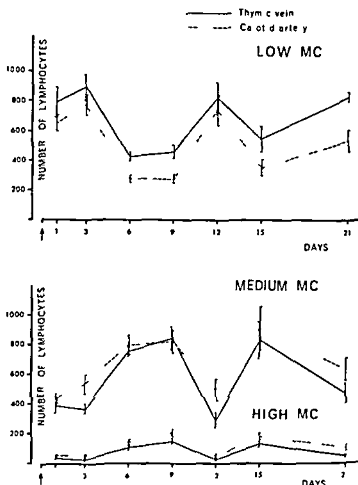


Fig 5

Number of lymphocytes with different mitochondrial content (MC) per mm³ of thymic vein and carotid artery blood of sham operated guinea pigs at different intervals after a single injection of pertussis vaccine

In the thoracic duct lymph the lymphocytes with different MC changed in incidence cyclically in essentially the same way as the blood lymphocytes (Fig 7) Thus the small lymphocytes decreased in incidence from day 3 to day 6 ($p<0.001$) increased from day 6 to day 12 ($p<0.001$) again decreased from day 12 to day 15 ($p<0.001$) and finally increased from day 15 to day 21 ($p<0.05$)

Antigen Treated Sham Operated Animals

The total number of blood lymphocytes was slightly but not significantly raised 3-21 days after the vaccine injection. The percentage of blood lymphocytes with different MC (Table 2) revealed cyclical changes after administration of the pertussis vaccine essentially as in the identically treated non-operated animals (Fig 3) The number

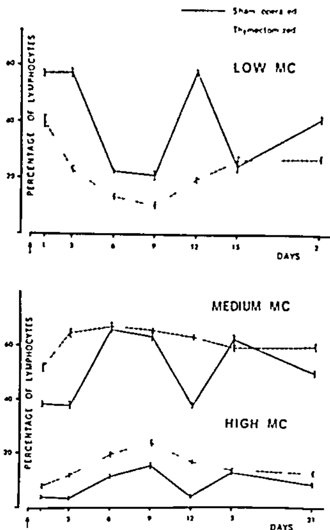


Fig 5

Percentage of lymphocytes with different mitochondrial content (MC) in carotid artery blood of sham operated and thymectomized guinea pigs at different intervals after a single injection of pertussis vaccine

of blood lymphocytes with different MC also changed in the same way as in the non operated animals Thus the number of "small" lymphocytes characterized by a low MC showed two maxima a first one 3 days after the antigen injection and a second one 12 days after it (Fig 4) The thymic vein contained more "small" lymphocytes per mm³ of blood than the carotid artery (Fig 4) This excess of "small" lymphocytes did not change during the immunization procedure The incidence of different classes of thoracic duct lymphocytes changed essentially as in the non operated animals (Fig 7)

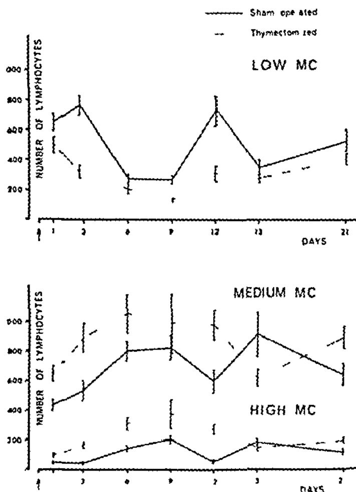


Fig. 6

Number of lymphocytes with different mitochondrial content (MC) per mm^3 of ear blood artery blood of sham operated and thymectomized guinea pigs at different intervals after a single injection of pertussis vaccine

Antigen Treated Thymectomized Animals

The total number of blood lymphocytes was slightly but not significantly raised 3-12 days after the vaccine injection. The cyclical changes in the percentage of blood lymphocytes with different MC (Table 2) recorded after injection of pertussis vaccine in non-operated and in sham-operated guinea pigs, did not occur in the thymectomized animals (Fig. 5 *cf* Fig. 1).

Throughout immunization the thymectomized animals had fewer "small" lymphocytes per mm^3 of blood than the corresponding non-operated and sham-operated animals (Fig. 6 *cf* Fig. 2). The increase in number of "small" lymphocytes 3 and 12 days after the antigen injection seen in non-operated and sham-operated animals failed to occur in the thymectomized guinea pigs (Fig. 6).

TABLE 3

Comparison Between Differential Counts of Thoracic-Duct Lymphocytes in Non Operated, Sham-Operated and Thymectomized Guinea Pigs at Different Intervals after a Single Injection of Pertussis Vaccine Mean ± SE

	Days after pertussis vaccination	No of animals	Classification of lymphocytes by mitochondrial content Lymphocytes in per cent						Average no of mitochondria/cell	p value of difference
			0 5	6 10	11 15	16 20	21 30	> 30		
Non operated	1	10	5±0 5	21±1 6	46±1 8	19±1 0	10±0 4	0 4+0 3	13 7+0 1	
Sham operated	1	10	5±1 7	21±1 0	42±0 8	22±1 1	9±0 3	0 9±0 1	13 9±0 2	
Thymectomized	1	10	2±0 7	14±1 0	45±1 8	27±1 7	11±0 4	2±0 4	15 2±0 2	p < 0 001
Non-operated	3	10	5±0 6	23±0 9	46±0 8	18±0 9	8±0 6	0 3+0 2	13 3+0 1	
Sham operated	3	10	5±0 6	17±0 8	43±1 7	24±1 2	11±0 5	0 3±0 2	14 1±0 1	
Thymectomized	3	10	0 2+0 1	6±0 4	40±1 4	33±1 1	18±0 6	3+0 5	17 1+0 1	p < 0 001
Non operated	6	10	1±0 3	11±0 1	42±1 2	29±1 1	16±0 8	3±0 4	16 2+0 2	
Sham operated	6	10	1±0 3	10±1 1	42±1 3	29±1 4	15±0 6	3±0 4	16 3+0 2	
Thymectomized	6	10	0±0	5±0 6	39±1 4	33±1 4	19±1 0	5±0 8	17 7+0 2	p < 0 001
Non operated	9	10	2±0 3	17±1 2	46±3 2	24±1 0	11±0 9	1±0 2	14 5+0 2	
Sham operated	9	11	0 8±0 3	9±0 7	41±1 2	30±1 0	15±0 5	4+0 5	16 6+0 2	
Thymectomized	9	10	0+0	6±0 7	35±1.3	31±1 0	22±1 2	5+0 8	18 0+0 2	p < 0 001
Non operated	12	10	6±0 5	24±0 8	43±1 5	18±1 4	9±0 6	0 7±0 3	13 4+0 2	
Sham operated	12	10	3±0 3	19±1 2	44±1 3	24±1 1	10±0 5	0 5±0 2	14 2±0 1	
Thymectomized	12	10	0 2±0 1	8±0 9	43±1 2	30±1 1	16±0 8	3+0 4	16 6±0 1	p < 0 001
Non operated	15	10	0 9±0.3	12±0 6	44±1 2	27±1 4	14±0 8	2±0 3	15 9+0 2	
Sham operated	15	10	1±0 4	9±1 0	40±1 2	32±1 3	15±0 8	3±0 3	16 4±0 2	
Thymectomized	15	10	0 8±0 3	11±0 9	42±1 9	29±1 5	15±0 5	3+0 4	16 3±0 2	
Non-operated	21	10	3±0 6	15±1 3	46±1 4	24±1.3	11±0 6	1+0 3	14 7±0 2	
Sham operated	21	10	3±0 3	14±0 7	42±1 3	27±1 2	13±0 7	2+0 3	15 2+0 2	
Thymectomized	21	9	1±0 3	10±0 9	41±1 1	31±1 5	14±0 4	3±0 5	16 2±0 2	p < 0 01

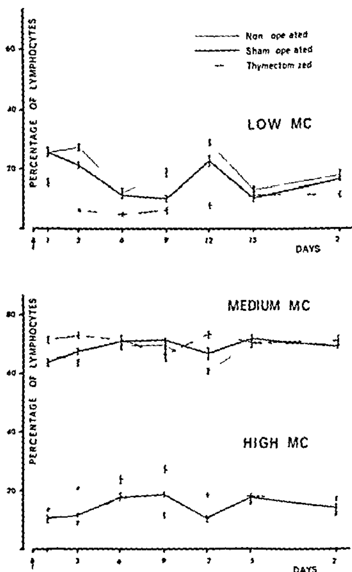


Fig. 7

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph of non-operated, sham-operated and thymectomized guinea pigs at different intervals after a single injection of pertussis vaccine

In the thoracic duct lymph the incidence of "small" lymphocytes was lower than in the guinea pigs with intact thymus (Table 3). The cyclical changes in incidence of "small" lymphocytes observed in the thoracic duct lymph of non-operated and sham-operated guinea pigs after a single injection of pertussis vaccine did not take place in the thymectomized animals (Fig. 7).

DISCUSSION

The possible participation of the thymus in an immune response to antigenic stimulation has been discussed in several symposia (*Good & Gabrielsen* 1964, *Defendi & Metcalf* 1964 *Wolstenholme & Porter* 1966) In the present study a single subcutaneous injection of pertussis vaccine in a low dose produced no significantly increased or decreased emigration of lymphocytes from the thymus when investigated by direct comparison between afferent and efferent thymic blood Unexpectedly, the experiment disclosed cyclical changes in incidence and number of different categories of lymphocytes in blood and in thoracic duct lymph and the absence of similar cyclical changes in thymectomized animals

In normal growing guinea pigs aged 30 50 days the number of lymphocytes with low MC (small lymphocytes) per mm³ of blood increased by about 25 cells a day whereas the number of lymphocytes with medium and high MC was almost constant (*Ernström & Larsson* 1966b) In the present study on the other hand administration of pertussis vaccine caused a decrease in the number of small circulating blood lymphocytes in both normal and thymectomized animals from 3 to 6 days after injection Concurrently the larger lymphocytes increased in number This may have been due to an antigen stimulated transformation of small lymphocytes into larger cells an interpretation which is in accordance with the observation of *Hirke et al* 1964 (*cf* phytohemagglutinin induced blastoid transformation of small blood lymphocytes *in vitro* *MacKinney et al* 1962 *Marshall & Roberts* 1963 *Sabesin* 1966) Another possibility is a disappearance of small lymphocytes from the blood and an independent increase in number of circulating large lymphocytes In the thymectomized animals the process caused almost total depletion of the smallest lymphocytes (those with 0 3 mitochondria per cell) 9 days after the antigen injection (Table 2) Thus in the non operated and sham-operated animals the consumption of small lymphocytes seems to have been counteracted by an output of thymic small lymphocytes preventing the total depletion of such cells in the blood

Between 9 and 12 days after the antigen injection the number of small blood lymphocytes again increased in all groups this increase being pronounced in the sham operated and less pronounced in the thymectomized animals Thus in the non operated and sham operated animals these lymphocytes seem to come from both thymus and non thymic lymphoid tissue

Following day 12 after the antigen injection a second rapid fall in the number of small lymphocytes and an increase in number of larger lymphocytes occurred in the non operated and sham operated animals but not in the thymectomized ones This second change may have been related to a secondary response to the original antigen injec

tion and to the generation time of the lymphocytes involved in the immune response (small lymphocytes→large lymphocytes→small lymphocytes→large lymphocytes). Enlargement of small lymphocytes after antigenic stimulation has been reported by *Porter & Cooper* (1962a, b), *Gowans et al* (1962) and *Rieke et al.* (1963). This implies that the second generation of small lymphocytes may be the cells anamnestic of the original antigen stimulation The cyclical changes which we observed in the blood lymphocyte population of guinea-pigs vaccinated with the polysaccharide antigen of *B pertussis* are strikingly similar to the periodical variations in the number of antibody-producing cells in the spleen after immunization with a lipopolysaccharide antigen from *E coli* (*Britton & Moller* 1966) Our results indicate that most lymphocytes circulating in the blood are involved in immune processes taking place in the organism It can also be stated that the absence of the thymus interferes with the normal changes in circulating lymphocyte populations occurring after pertussis vaccination

SUMMARY

Three groups of male guinea-pigs, non-operated, sham-operated and thymectomized, were investigated The lymphocyte populations of carotid-artery and thymic-vein blood and thoracic-duct lymph were studied at different intervals after a single subcutaneous injection of pertussis vaccine The lymphocytes were classified by their mitochondrial content

In the non-operated and sham-operated animals, the number of "small" lymphocytes with 0 10 mitochondria per cell were found to change cyclically, with minima on day 6 and 15 after the antigen injection, when the larger lymphocytes were maximally raised in number This periodicity did not appear after thymectomy In the thymectomized animals, the number of "small" lymphocytes was reduced, but tended to be restored at the end of the experimental period

The thoracic-duct lymphocytes were found to undergo similar changes to those in the blood lymphocytes, although they were less conspicuous

The normal excess of "small" lymphocytes in the thymic-vein blood as compared to the carotid-artery blood persisted throughout the experiment with no significant increase or decrease

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Acta path et microbiol scandinav 69 610 611, 1967

BRIEF REPORTS

MIXED CRYOIMMUNOGLOBULINS

By *Odd Wager, Jorma A Räsänen Allan Lassus* and *Kimmo K Mustakallio*

By screening techniques cryoglobulins have been found by us in 15 per cent of syphilitic sera (7), in 37 per cent of CBFP sera (4) in 53 per cent of SLE sera (5) in 40 per cent of mononucleosis sera (10) and in 3 per cent of sera from patients with various dermato-venereological diseases (8)

In the present communication some immunological and clinical data of 19 patients with mixed cryoimmunoglobulinemia are reported

As seen from the table three out of the total of 19 isolated cryoglobulins were composed of IgA and IgG immunoglobulins Two of the latter contained in addition to IgA and IgG small amounts of β_1A a moiety of the third component of complement Immunological studies and case reports of these three patients with IgA IgG cryoglobulinemia will be given elsewhere (9)

All the remaining 16 cryoglobulins were composed of IgM and IgG One of them also contained β_1A Four patients had a clinical picture characterized by purpura arthralgia Raynaud s phenomenon and rheumatoid factor (RF) activity of their serum (2 3) Three patients had SLE (6) two were acute nephropathies probably induced by drugs four patients had syphilis in various stages of the disease and three were cases of mononucleosis Two of the latter had a typical Paul Bunnell positive infectious mononucleosis whereas the third was a patient with cytomegalo virus mononucleosis (1)

Most of the isolated cryoglobulins possessed a strong anticomplementary effect, like that shown under the same experimental conditions by known specific precipitates of human and rabbit origin The anticomplementary activity of the cryoglobulins was abolished by treatment at +56° C for 30 minutes Dissociation at pH 3 5 and Sephadex 200 gel filtration of one of the IgM IgG cryoglobulins (TV) resulted in separation of the IgM and IgG components both devoid of cryoprecipitability Recombination of the two components restored the cryoprecipitability

The strong anticomplementary activity the presence of β_1A in three of the mixed cryoglobulins and the dissociation and reprecipitation patterns of one of the cryoglobulins studied suggest that the mixed cryoimmunoglobulins are circulating antigen antibody complexes The simultaneous occurrence of a number of recognized autoantibodies (antinuclear antibodies cold agglutinins rheumatoid factor activity) in several instances gives further support to this hypothesis

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Received 22 iii 67 from *the Municipal Bacteriological Laboratory Aurora Hospital Helsinki* and *the Department of Dermatology and Venereology University Central Hospital Helsinki Finland*

This study was supported by Grant AM 3026 from the *National Institute of Arthritis and Metabolic Diseases U S Public Health Service* and by grants from the *Sigrid Jusélius Foundation* and the *Yrjö Jahnsson Foundation Finland*

Patient	Waaler Rose Titre	Latex Test	Antinuclear Antibody MLI	Antinuclear Antibody HLI†	CH Aggl Titre	Conc of Cryogl (µg/ml)	CF Activity (µg required for)	Composition	Remarks
			Whole Serum			Isolated Cryoglobulin			
TS						100	9	IgA IgG β1A	Purpura Haematuria
CP						250	18	IgA IgG β1A	Purpura Haematuria
AB						2[illegible]0	18	IgA IgG	Raynaud's phenomenon Cold Urticaria Arthralgia Latent Syphilis
HB	10[illegible]0	+				5000	>140	IgM IgG	"Purpura Arthralgia Raynaud's phenomenon HB syndrome"
IN	500	+++	+			1500	215	IgM IgG	
IA	250	+++				25	>75	IgM IgG	
IR	128	+++				[illegible]90	14	IgM IgG	
AK	10[illegible]0	++	+	+	128	550	50	IgM IgG β1A	Systemic Purpura
AT	1[illegible]8	++	+	+		220	55	IgM IgG	Erythematous
MR	128	+	+	+		2	6	IgM IgG	
JS	1[illegible]8	*		+	64	175	15	IgM IgG	Nephropathy (Indirectly by Butazolidine*
SS	[illegible]	+	+		128	40	9	IgM IgG	Sulphonamide?)
KR	64					440	27	IgM IgG	Secondary Syphilis
FI					32	[illegible]0	35	IgM IgG	Secondary Syphilis
MK						100	12	IgM IgG	Syphilitic Osteitis
LW		+?	+	+		30	7	IgM IgG	Latent Syphilis
AI		—	+	+	512	275	12	IgM IgG	Infectious Mononucleosis
KC		—	+?		128	1[illegible]0	80	IgM IgG	Infectious Mononucleosis
JI	64	+?			2000	1.5	0.8	IgM IgG	Cytomegalovirus Mononucleosis

MLI Mouse liver cryostat section as antigen (Indirect IFA technique)
† HLI Human peripheral leucocytes as antigen (Indirect IFA technique)

Acta path et microbiol scandinav 69 612-613 1967

THE ORDER OF APPEARANCE OF REACTIVITY TO TREPONEMAL AND LIPOIDAL TESTS IN EARLY SYPHILIS

By A *Lassus* K K *Mustakallio* K *Aho* and T *Putkonen*

Adequate information of the order of appearance of reactivity to serological test for syphilis can be gained only from untreated syphilitic patients with known date of the infection

Sera from 76 such male patients—62 with darkfield positive primary and 14 with secondary syphilis—were tested with fluorescent treponemal antibody absorption (FTA ABS) (3) treponema pallidum immobilization (TPI) modified Reiter protein complement fixation (RPCF), Kolmer cholesterol Wassermann (ChWaR) VDRL, and Kahn tests The FTA-ABS test was performed according to the provisional technique published in 1965 by the Venereal Diseases Research Laboratory of the Communicable Diseases Center, Atlanta The RPCF test was carried out with a microtechnique (2) using 2 units of complement The other tests were performed according to the U S Public Health Service's Manual for Serologic Tests for Syphilis

In the table the tests are presented in decreasing order of sensitivity in early syphilis The FTA ABS test became reactive in practically all cases within 45 days from the infection the Kolmer and VDRL tests within two months and the other tests three months after infection Within the first six weeks 6 patients showed reactivity only in the FTA ABS test and 4 only in some of the lipoidal tests

TABLE

Appearance of Reactivity to Serological Tests for Syphilis in 76 Cases with Known Date of Infection (Seropositive Cases / Total)

Days from infection		FTA ABS	Kolmer	VDRL	Kahn	ChWaR	RPCF	TPI
	30	3/11	4/11	1/11	1/11	0/11	0/11	0/11
31	45	18/20	14/20	11/20	10/20	6/20	4/20	2/20
46	60	20/20	19/20	17/20	15/20	11/20	7/20	4/20
61	90	15/15	15/15	14/15	13/15	11/15	8/14*	7/15
91		10/10	10/10	10/10	10/10	10/10	10/10	10/10

* One serum was anticomplementary

Among the 62 patients with primary syphilis 84 per cent displayed reactivity to FTA ABS 77 per cent to Kolmer 63 per cent to VDRL 56 per cent to Kahn 39 per cent to ChWaR 28 per cent to RPCF and only 15 per cent to the TPI test In the 14 cases with secondary syphilis all tests were reactive except the micromodification of RPCF which was negative in two patients

Deacon and his coworkers (1) have recently reported that the FTA ABS test gave a positive reaction in 86 per cent of their patients with untreated syphilis 76 per cent showed reaction to the VDRL and 63 per cent were reactive in the TPI test Their patients were not classified according to the duration of syphilitic infection but since a greater proportion of them were reactive to the VDRL test it is probable that the infection was of longer duration on the average in their cases than in our

Received 17 iii 67 from the *Department of Dermatology and Venereology University Central Hospital* Snellmaninkatu 14 Helsinki 17 Finland and from the *State Serum Institute* Helsinki Finland Supported by grants from the *Sigrid Jusélius Foundation* Helsinki Finland

cases. Th s may explain th e lifferences in the percentages f reactive tests being particularly great in th e sl west test the TPI

In conclusi n the FTA ABS test letects syph ilis earlier than th e ther treponemal te ts an l frequently even earlier than the lipo l lal te ts.

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AUTOIMMUNE PHENOMENA IN PATIENTS WITH OLD TREATED SYPHILIS NONREACTIVE TO THE TREPONEMA PALLIDUM IMMOBILIZATION TEST

By *A Lassus K K Mustakalli* an l *T Putk nen*

Syphilitic infection in luces immun l gical aberrations f the a t imm ne type including pro lucti n f antilip idal reagins (1) l th ermic col l haem lysine (8) cryoglol ulins (5 an l rhe matoid fact rs (5) On th e ot er hand bi l gical false p sitive (BFP) antilip oi lal reagins may devel p years bef re manife tati n f cer tain autoimmune di eases such as systemic lupus eryth emat us (SLE) or rhe m atoid arthritis (6) In st dies f the chr nic BFP phen menon the flu rescent tre

wh ha l been treate l wit heavy metals 15 to 53 years ag f r either primary or secondary syph ilis Twenty three f the patients were remitted because f s me dermat sis an l 13 f r the foll w i g f syphili

In the tal le the 36 patients are grou pe l acc r ling t their reactivity to the TPI and the lip i lal (STS test incl l ng the VDRL Kahn ch lester l Wa ermann and K lmer te ts

N less than 24 out f th e 36 patients were n nreactive to the TPI te t Sera from 15 TPI negative cases were availal le f r the FTA ABS test which vas p iti e in 11 cases

Pr se l a t immune lisease rh eumat id fact r activity r cryogl bul ns were f un l only am ng the patients with persi tent reactivity t s me f the l p lal tests (STS + Group I and III In the TPI negati e Gr p I there v as evidence of auto immune di r ler in 8 ut of 11 cases an l in the TPI p s tive Gro p III in 5 o t of 10 cases In the former gr up one patient de el pe l def nite SLE ne lefinite ra several years af er the treatm nt

fect on In the TPI positi e Group II def nite rh u at d art it s deve oped after syph litic f ction in one female patient an l cryogl l al nemic pur ra in one male pat ent

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TABLE
Characteristics of 36 Patients with Old Treated Syphilis

Group		Number of cases	Females	Mean age in years	Years from treatment (mean)	FTA-ABS (+/total)	Proved autoimmune disease	Rheumatoid factor (+/total)	Cryoglobulins (+/total)
I	TPI — STS +	11	7	59	36	6/8	4/11	5/10	3/9
II	TPI — STS —	13	3	56	31	5/7	0/13	0/6	0/6
III	TPI + STS +	10	5	59	36		2/10	3/8	3/7
IV	TPI + STS —	2	1	48	30		0/2	0/0	0/0

These results strengthen the evidence of the autoimmune nature of the antilipoidal reagins in syphilis. Whether or not syphilitic infection could trigger an autoimmune disease in predisposed subjects remains to be determined.

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Acta path. et microbiol. scandinav. *69* 614-615 1967

ANTINUCLEAR FACTORS IN SERA FROM CHRONIC FALSE POSITIVE SEROREACTORS FOR SYPHILIS

By *K. K. Mustakallio, A. Lassus and J. A. Räsänen*

Sera from 51 chronic biologic false positive (CBFP) reactors (4, 5), all negative both to treponema pallidum immobilization and fluorescent treponemal antibody absorption tests (2), were examined with the indirect antinuclear factor fluorescent antibody (ANF-FA) technique using both cryostat sections of mouse liver and smears of human peripheral leucocytes as the source for nuclei (1). One serum sample

Received 20.11.67 ...
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st re l deej fr zen f r tw years n the average was availat le fr m each of the 51 CHEP react rs Nine of these t a l lefinite an l 7 pr l al le systemic lup s eryt emat s s (SLE) a lhering to the criteria pre ente l el ewhere 3 an l 4 lefinite an l 3 pr l al le rhet mat l l artl ritis acc r ling t the ARA criteria

Six out of tl e 9 patients with lefinite SLE an l 2 f the 7 patients with pr l al le SLE showe l a po itive ANF FA test In one of the 2 patients with pr l al le SLE the test was p sitive nly with h man leu c cytes.

Am ng tl e remaining 35 CBFT react rs sh wing neither LE-cells n r DNA anti b dies 4 ha l a p itive ANF FA te t Three f these ha l pr l al le rl e matoi l arthritis. They were all females, 31 34 an l 67 years ol l respectively It will be interesting t see whether r n t they level p tl e rheumat l l variant of SLE (cf 3) The f rtl ANF FA p sitive CBFT react r was an asympt matic 54 year-ol l man The 4 CBFT reactors with lefinite rhe mat l l artl ritis an l 2 react rs with p rpura showed a negative ANF FA test

In c nclusi n the ANF FA test p ssesse l a fairly good li criminat ry p wer for SLE among tl e 51 CBFT react rs stu lie l

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Acta path. et micr l i l Scan linav 69 615 616 1967

ANTINUCLEAR FACTORS IN PSORIATIC ARTHRITIS

By *K K Mustakall o A Lassus an l J A Räsänen*

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n itive ANF FA test
antinuclear factors
er arthr pathies as

sociated with psoriasis

Both cry tat secti ns f m u e l er an l smears of human le c cytes serve l as tl e s ur f n clei for the in lirect ANF FA te t (2 The flu re cein conjugate l h rse anti man gl l l n serum c ntaine l antib lies f r human IgG IgM and IgA The p riat c arthr pathies were classified acc r ing t r entgenol g cal changes

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asis an l rheumat i l arthritis like X ray changes with nt rheumat 1l fact r activity in the serum

No one of the 10 psoriatics with osteoarthritic changes and of the 39 patients with uncomplicated psoriasis vulgaris sh we l in their sera antinuclear fact rs demonstrable with the ANF FA test

In conclusion sera from patients with ps riatic arthritis sh w a m ch lower incidence not only of rheumatoid factors (4) but also antinuclear factors dem nstrable with the immunofluorescent test as compare l with rheumat il arthritis sera

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Acta path et micr l l l scandinav *69* 616 617 1967

PAPAIN FRAGMENTS OF HUMAN G MYELOMA GLOBULINS WITH SEROLOGICAL ACTIVITY

By *Olle Zettervall*

Some cases of human sera containing both G myel ma gl bulins (M-components) and exceptionally high antibody like activity (*Waldenström* 1961 *Kritzm & Cabau* 1964 *Waldenström et al* 1964 *Marsa & Kjems* 1965 *Wanblad* 1966 pers nal c mmunicati n *Kronvall* 1967 unpublished work) are now kn wn In s me cases there is evidence that the ser l gical activity is c nfined to the M-c mp nent (*H d n & Cab r* 1964 *Mansa & Kjems* 1965 *Zettervall et al* 1966 *Kronvall* 1967 unpublished w rk

This rep rt deals with three of these M c mp onents tw f wh ch have high antistrept lysin O AST activity (*Zettervall et al* 1966) and one with c nsiderable ASTA activity *Wanblad*s case)

M-c mp nents were btaine l by ion exchange chromatography f the sera The AST titres f the tw AST active M-c mp nents was 8000 and 19 00 U per ml respectively at a pr tein c ncentrati n f 10 mg/ml For the third M mp nent the ASTA titre was 00 U per ml at the same protein c ncentration The n rm al gamma gl bulin preparati ns f the sera sh we l negligible r n ... digesti n f the M-c mp nents was perf rmed acc rding t *Hsia & Putnam* (1961) meth d with slight m dificati ns The reacti n was st pped with para-chl r mercuribenz ate The three digests were characterized in several wa s In all cases thin layer gel filtrati n in Sephadex G 100 Superfine sh we l that m st of the material giving pr tein staining was f l wer m lecular size than intact γG gl bulin

Zone electr ph r sis f the digests dem nstrated the presence f tw c mp nents n t f und in undigested material One c mp nent m ve l faster and the ther slower than the parent M c mp nent Immun electr ph retical analysis f the digests was

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performed using rabbit antisera against pooled normal human γG-globulin, against the individual undigested M-components and against human light chains. In all cases the results indicated that the fast and slow electrophoretical components consisted of Fc and Fab fragments respectively. The proportions of Fab to Fc fragments in the three digests were calculated from light absorbency measurements of eluates from protein stained electrophoresis strips. Determined in this way the quotient Fab/Fc was 4.3 or lower.

To visualize the distribution of serological activity on cellulose acetate electrophoregrams of M-components and their papain digests blood agar plates containing streptolysin O and α-staphylolysin respectively were used. Part of the electrophoresis strips were placed in contact with the plates for a suitable time allowing the proteins to diffuse into the gel and then removed. When AST activity was to be determined the inactive oxidised streptolysin-O was then activated by application on the plate of a filter paper soaked in pyrosulphite solution. Details of this technique will be given elsewhere (*Zettervall*, to be published). Fractions were localized by [illegible]

[illegible]

material was similarly analysed a maximal inhibition of lysis occurred only at the site of the M-component, i.e. nearer the anode than the haemolysis inhibition zone from a digest applied at the same electrophoresis strip.

The patterns were uniformly reproducible and their correspondence to the patterns on the stained portions of the electrophoresis strips was good. These experiments show that the Fab fragments from the three M-components have antibody activity whereas the Fc fragments seem inactive.

Studies in progress on the two AST active M-components suggest that streptolysin-O is actually bound by these proteins. Hence the serological activity of their Fab fragments reflects probably also such binding. Heavy and light chains from [illegible]

[illegible]

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Acta path et microbiol scandinav 69 618 619 1967

TRANSIENT MODIFICATION OF THE HeLa CELL ORGANELLES FOLLOWING REDUCTION OF GLUCOSE SUPPLY

By *Margrete Willoch* *

When the glucose concentration of the nutritive medium of HeLa cells is cut down from the standard 100 mg per cent to 6 mg per cent the submicroscopic morphology of the cell surface is permanently changed in the direction of fewer and less complex cytoplasmic protrusions and smooth cell borders (*Willoch* 1967) This change is interpreted as a reduction of cell surface area related to a reduced glucose metabolism

The present note describes the reaction of cytoplasmic organelles to a limitation of accessible glucose

Material and Methods

HeLa cells strain S3 were kept in the medium F2a of *Puck Cieciura & Fisher* (1957) which contains 100 mg per cent glucose Experiment medium containing 6 mg per cent glucose was prepared according to the same recipe the addition of glucose being omitted and the human serum fraction being treated with baker's yeast and filtered through a bacterial filter before being added to the medium

Cells were fixed weekly first time 3 days after transfer to experiment medium The cultures were fixed in buffered 3 per cent glutaraldehyde postfixed in buffered 1 per cent OsO_4 and embedded in Vestopal W Thin sections were cut on an LKB Ultrotome and examined in a *Zeiss* EM 9 electron microscope

Results

The mitochondria Golgi region lysosomes and endoplasmic reticulum of HeLa cells from long term cultures in either of the two media used are all of little specific appearance

Mitochondria are elongate occasionally branched about 0 25 microns wide with a rather sparse content of longitudinal or transversal cristae which frequently traverse the total width of a scanty matrix

The extensive Golgi apparatus consists of several stacks of flat cisternae enclosing a vacuolated area and surrounded by numerous small vesicles

Lysosomes are most frequently seen in the form of multivesicular bodies

The endoplasmic reticulum appears as scanty unsystematically arranged short or long profiles without conspicuous granulation

3 days after transfer to experiment medium the cytoplasmic organelles have the following characteristics

Mitochondria are of unchanged size Their matrix is very dense the cristae being difficult to distinguish

The vacuolated appearance of the Golgi region is more pronounced owing to a dilation of the dictyosomal cisternae

The lysosomes increase markedly in number They appear as heterogeneously dense bodies mostly containing membrane elements

There is a strong development of the endoplasmic reticulum which tends to be arranged in relatively dense parallel or whirlpool like patterns Their granulation is markedly increased The occurrence of granula in the cytoplasm is also more conspicuous

These structural modifications subside after some 10 days the cytoplasm regaining the appearance of long term cultures

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Received 21 [illegible] From the Department of Pathology Sentralsykehuset i Trondheim Norway Head Erling A Mylius M D

* Fellow of the Norwegian Cancer Society

Discussion

Being of a purely morphological nature the present observations do not offer the opportunity of an unambiguous functional interpretation.

Transition to experiment medium does not involve detectable changes in long term growth rate or weekly growth pattern of the HeLa cells even immediately after transfer. It thus seems unlikely that the observed organelle transformations represent an effort to maintain a normal reproductive capability.

Whereas a reduction in the glucose supply of HeLa cells induces a corresponding reduction in the total amount of glucose metabolized (*Eagle et al.*, 1958) this treatment seems to leave their glycolysis-respiration ratio undisturbed (*Eagle et al.* 1958, *Abdel Tawab et al.* 1959). The present structural modifications probably indicate a quantitative rather than a qualitative shift in cellular functions.

The involvement of all these organelles suggests that respiration, protein synthesis, and digestive processes are all affected by the change of nutritive conditions. The simultaneous appearance and short duration of the transformations also indicate that they are in some way related to each other and that they may represent a shock and/or functional relaxation reaction to the change in environment.

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LIGAND BINDING SITES FOR STREPTOLYSIN O AND STAPHYLOCOCCAL PROTEIN A ON DIFFERENT PARTS OF THE SAME MYELOMA GLOBULIN

By *Göran Kronvall*

Dammacco & Clausen (1966) reported an IgG myeloma globulin preparation precipitating staphylococcal protein A as well as an antigen from *Diplococcus pneumoniae* type EA 1a. Dammacco & Clausen were apparently inclined to ascribe the anti-protein A activity to the contaminating normal gamma globulin in the myeloma globulin preparation.

I have tested 44 myeloma sera containing IgG myeloma globulins for antistreptolysin O and for precipitins of protein A[1] (*Löfkvist & Sjöquist* 1963, *Lofkvist* 1966) from *Staph. aureus*. Two sera showed exceptionally high titres of antistreptolysin O (AST 280,000 and 70,000 IU per ml respectively). On immunoelectrophoresis these 2 myeloma sera precipitated protein A like several of the other myeloma sera with the formation of an arc corresponding to the myeloma band. The gamma globulin concentrations of myeloma serum dilutions still giving a precipitate against protein A in agar gel diffusion tests were lower than that of a pool of normal human sera. Serum from patient M. P. with an AST titre of 280,000 IU per ml was investigated further.

Received 13.iii.67 from the Department of Medical Microbiology, University of Lund, Lund, Sweden.

Head: Professor Rune Grubb.

[1] Purified protein A was kindly supplied by Dr. T. Löfkvist, Lund.

The myeloma globulin present in the serum M P at a concentration of 5.3 g per 100 ml was typed Gm(—1 —2 4 —5) (nomenclature according to WHO 1965) with light chains of *κ* type The myeloma globulin was purified in two steps by gel filtration and zone electrophoresis (*Killander* 1963) On immunoelectrophoresis the *preparation showed no traces of normal gamma globulin when tested at 2.9 g per* 100 ml Papaindigestion and separation of the fragment were performed according to *Porter* (1959) Heavy and light chains were prepared and separated according to *Olins & Edelman* (1964)

The AST titres of the purified myeloma globulin and of the Fab fragment was about 22 000 IU per ml at a concentration of 10 mg protein per ml The corresponding AST titre of the light chain preparation was 1 700 IU per ml whereas the Fc fragment and the preparation of heavy chains did not show any significant activity The streptolysin O combining site has been located on the Fab fragment for two other myeloma globulins with high AST activity (*Zetterwall* unpublished work)

On immunoelectrophoresis the myeloma globulin preparation like the original serum precipitated purified protein A from *Staph aureus* with an arc suggesting a monoclonal precipitating antibody as described earlier by Dammacco & Clausen (1966) In the recent study by *Forsgren & Sjöquist* (1966) the combining site for staphylococcal protein A as shown by inhibition experiments was said to be located on the heavy chains and on the Fc fragments of normal human gamma globulin and myeloma globulins Precipitation of protein A by the purified myeloma globulin M P was inhibited by the heavy chains but not by the light chains or by the Fab and Fc fragments The papain digestion technique resulted in a more extensive degradation than w [illegible] explain why the preparation [illegible] the protein A combining site [illegible] presumably in that part which is broken down to small peptides [illegible] papain digestion

According to *Jensen* (1959) the agglutination of *Staph aureus* by normal human sera is an effect of the combination between protein A on the surface of the cocci and corresponding antibodies To ascertain whether the combining sites for streptolysin O and protein A were located on the same molecules or whether the myeloma globulin was heterogeneous in this respect absorption by living staphylococci was performed Staphylococci Cowan type I phage type 52 from an 18 hour culture grown on solid medium were washed in saline 4×10^{12} bacteria were added to 1 ml of the myeloma globulin and the Fab fragment preparation containing 50 mg protein respectively After 2 hours at room temperature the tubes were centrifuged and the supernatants tested for AST activity Controls without added bacteria were included

AST Titre of Purified Myeloma Globulin and Fab Fragment after Absorption by Staph aureus The Titre Is Expressed as IU per ml at a Protein Concentration of 10 mg per ml

Myeloma globulin		Fab fragment	
absorbed	not absorbed	absorbed	not absorbed
400	22 000	11 200	17 600

As shown in the table the AST activity of the purified myeloma globulin was reduced to less than 2 per cent after absorption by staphylococci whereas the AST activity of the Fab fragment was only slightly reduced

These absorption experiments indicate that ligand binding sites for streptolysin O and staphylococcal protein A are located on different parts of the same myeloma globulin molecules These immunoglobulin molecules may thus react with streptolysin O and with protein A although the latter proteins do not in a ss react in the ordinary sense of this term At least some immunoglobulin (o m l c) may thus have complementary sites for more than one structure In this study both structures being of bacterial origin

Rf r n et Dammis on F C e Lfuven J Acta Med Scand 1 5 a 765 1968 *For j n C C Sjō ju st J* J Immun 1 gy 9 872 82? 1964 *Jensen K* Thesis Munksgaard K penhagen 1959 p 34 *K Han fre J* Acta soc Med Ups al 66 239 ?11 1963 *Kr nwall C* Va Sang 10 203 313 196 *Leffler T & Sjō qu st J* Int ?11 1963 *Kr nwall C* ... Arch Allergy ?3 249 20 1963 *Fler t T* Int Arch All rgy ?3 240 2 3 1963 *Potter R R* ... *Anderson D F & Fleiman C M* J exp Med 119 ?53 85a 1964 ... Chem J 5 119 126 1959 World Health Organisation Bull Wld Hlth Org 3? ?1 ? 1 1965 *Zetterwall O* Unpubl l ed w rk

Acta path et micr bi l scand nav 69 631 673 1967

PHYLOGENETIC VARIATIONS IN THE VASCULAR PERMEABILITY OF PERIPHERAL NERVES TO SERUM ALBUMIN

A Preliminary Report

By *Yngve Olsson*

In a recent study on the permeability of vasa nervorum in rat peripheral nerves a fluorescent protein tracing technique introduced by Klatzo and coworkers 1962 for studies of the blood brain barrier was applied (*Olsson 1966 1967*). Contrary to the epineurial and perineurial blood vessels the endoneurial vessels did not permit passage of intravenously injected fluorescent labelled albumin through their walls. The extravasated albumin in the surrounding nerve sheaths was also prevented from diffusing into the endoneurium by the innermost parts of the perineurium. Thus the penetration of serum albumin into the endoneurium of normal rat peripheral nerves seems to be controlled by the joint action of the endoneurial vessels and the perineurium.

In traumatized nerves a heavy inflow of serum albumin mainly derived from leaking endoneurial vessels occurs in the endoneurium (*Olsson 1966*). For further studies of the vascular response in other peripheral neuropathies it is sometimes desirable to choose other animal species than rats since this animal is highly resistant to certain experimental neuropathies. Knowledge of the normal vascular permeability in different species is prerequisite for such studies. In the course of a study during which the permeability of vasa nervorum to serum proteins was outlined phylogenetic differences in the permeability of endoneurial blood vessels were observed. The findings are briefly reported here.

Material and Methods

Data concerning the examined animals are summarized in Table I.

Differences in the vascular permeability were ...
microscopic tracing of intravenously inj ...
rescein isothiocyanate (FITC) (*Klatzo et* ...
(*Steinwall & Klatzo 1965*). The technique ...
conjugates in thin frozen sections fixed in formalin due to the intense green fluorescence of FITC and the red fluorescence of EBA.

Each animal received one intravenous injection of either FITC or EBA. They

Received 14.III.67 from the *Neuropathological Laboratory Department of Pathology I University of Göteborg Sweden*

[1] Supported by grants from the *Swedish Multiple Sclerosis Society*

The myeloma globulin present in the serum M P at a concentration of 5 3 g per 100 ml was typed Gm(—1 —2 4 —5) (nomenclature according to WHO 1965) with light chains of λ type The myeloma globulin was purified in two steps by gel filtration and zone electrophoresis (*Killander* 1963) On immunoelectrophoresis the preparation showed no traces of normal gamma globulin when tested at 2 9 g per 100 ml Papaindigestion and separation of the fragment were performed according to *Porter* (1959) Heavy and light chains were prepared and separated according to *Olins & Edelman* (1964)

The AST titres of the purified myeloma [illegible]

a [illegible] protein A from *Staph* [illegible] h an are *suggesting* (1 [illegible] ammacco & Clausen st [illegible] combining site for ca [illegible] chains and on the Fc fragments of normal human gamma globulin and myeloma globulins Precipitation of protein A by the purified myeloma globulin M P was inhibited by the heavy chains but not by the light chains or by the Fab and Fc fragments The papain digestion technique resulted in a more extensive degradation than what is generally reported (*Kronvall* 1965) and this may explain why the preparation of Fc fragments did not show any inhibition Thus the protein A combining site of myeloma globulin M P resides in the heavy chains presumably in that part which is broken down to small peptides by prolonged papain digestion

According to *Jensen* (1959) the agglutination of *Staph aureus* by normal human sera is an effect of the combination between protein A on the surface of the cocci and corresponding antibodies To ascertain whether the combining sites for streptolysin O and protein A were located on the same molecules or whether the myeloma globulin [illegible] was performed [illegible] culture grown [illegible] to 1 ml of the [illegible] µg protein [illegible] After 2 hours at room temperature the tubes were centrifuged and the supernatants tested for AST activity Controls without added bacteria were included

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... *Dumonde D. C., Glynn L. E.*: ... *Ahlqvist J.*: Acta Med. Scand. 179, 755–769, 1966. ... *Olsson Y. & Sjöqvist J.*: J. Immunol. ... 9, 827–82, 1966. *Jensen K.*: Thesis, ... gaard, Köpenhagen 1959, p. 35. *Klinger J.*: Acta Soc. Med. Upsal. 69, 230 ... *Kr. ...*: Acta Soc. Sang. 10, 303–313, 196... *Ulfh... & Sjöqvist J.*: Int. Arch. Allergy 23, 259–30, 1963. *Ulfh... T.*: Int. Arch. Allergy 29, 240–253, 1966. *Orr D. P. C. & Feldman C. M.*: J. exp. Med. 119, 789–815, 1964. *Porter R. R.*: ... J. 73, 119–126, 1959. World Health Organization: Bull. WHO, 1964. Org. 33 ... 196... *Zetterwall O.*: Unpublished work.

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In traumatized nerves a heavy inflow of serum albumin mainly derived from leaking endoneurial vessels occurs in the endoneurium (*Olsson* 1966). For further studies of the vascular response in other peripheral neuropathies it is sometimes desirable to choose other animal species than rats since this animal is highly resistant to certain experimental neuropathies. Knowledge of the normal vascular permeability in different species is prerequisite for such studies. In the course of a study during which the permeability of vasa nervorum to serum proteins was outlined phylogenetic differences in the permeability of endoneurial blood vessels were observed. The findings are briefly reported here.

Material and Methods

Data concerning the examined animals are summarized in Table I.

Differences in the vascular permeability ... were ... at ... fl... mier se ... tracing of intravenously injected serum albumin ... tagged with fluoresceinisothiocyanate (FITC) ... *Klatzo et al.* 1962 ... with ... Evans blue EBA (*Steinwall & Klatzo* 1965). The technique ... the possibility of tracing the conjugate in thin frozen sections of unfixed formalin ... the intense ... fluorescence of FITC and the red fluorescence of EBA.

Each animal received one intravenous injection of ... FITC or EBA. The

TABLE 1

Data about the Examined Animals the Tracers Used and a Summary of the Results (+ indicates areas in which the blood vessels allow the passage of the tracer – the opposite)

Species	Number	Body weight (grams)	Tracer	Circulation time (hrs)	Permeability of vasa nervorum	
					Endoneurium	Epi perineurium
Rat	25	150–350	FLA, EBA	1/4–24	–	+
Guinea pig	9	315–460	FLA, FBA	1/4 6	+	+
Rabbit	5	1400–1600	FLA FBA	2 6	+	+
Hen	4	1300–1900	EBA	2–6	+	+
Cat	4	1300–2700	FLA	5/60 24	*	+

* The two cats examined five minutes after the injection did not show signs of albumin extravasation contrary to the other two cats examined twenty four hours later

were killed at various times thereafter and the two sciatic nerves were removed for examination The details of this technique have previously been described (*Olsson* 1966)

Results

Subsequent to injection of either FLA or FBA an intense fluorescence was visible in the lumen of vasa nervorum of all animals An extravasation of labelled was also observed both in the epineurium and the perineurium Here an infiltration of albumin increasing with time after injection suggestive of a slow transvascular passage of albumin was observed

There were obvious species differences in the vascular permeability to serum albumin in the endoneurium of the nerves Whereas the rats did not show transvascular passage of the fluorescent albumin in the endoneurium (Fig 1) this was regularly observed in rabbits guinea pigs hens and cats when the animals were allowed

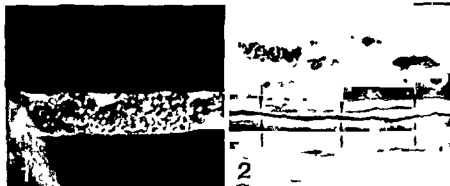


Fig 1 Fluorescent albumin confined to the lumen of an endoneurial blood vessel of a rat sciatic nerve There is no fluorescence outside the blood vessel

Fig 2 Longitudinal section through a guinea pig nerve after intravenous injection of FLA Note the cellular uptake and the diffuse extravasal fluorescence of FLA in the epineurium (upper part) the accumulation of FLA in the perineurium (upper arrows) and the diffuse faint fluorescence in the endoneurium (lower part) with accentuation in the subperineurial space (lower arrows)

to survive for six hours or more after the injection (Fig. 2). In these animals varying amounts of fluorescent albumin were present in the endoneurial interspaces. The penetration rates seemed also to differ in animals in the latter group but the present material is insufficient to provide quantitative aspects on this problem.

An interesting finding frequently encountered in rabbits, guinea pigs and hens was the accumulation of fluorescent albumin in a narrow zone immediately beneath the perineurial epithelium (Fig. 2). This finding proves that the subperineurial space often seen in histological sections is not exclusively an artefact but a preformed tissue space in communication with the endoneurial spaces.

Discussion

The present findings demonstrate that the permeability of vasa nervorum to serum albumin shows species variations. Previously *Waksman* (1961) observed in a study on the permeability of vasa nervorum to diphteric toxin and various dyes that also the permeability to these substances show species differences. He considered the permeability properties of vasa nervorum to be of considerable significance for determination of the distribution of nerve fibre lesions in peripheral neuropathies caused by blood-borne agents. Waksman was of the opinion that one reason for the resistance of rabbit peripheral nerves to experimental diphteric neuritis might be that the circulating toxin cannot pass from the blood into the endoneurium of rabbit peripheral nerves. However, the present study has shown that the vasa nervorum of normal rabbit nerves permit the passage of albumin across their walls. One reason for these diverging results might be that diphteric toxin and albumin penetrate at different rates across the vascular walls. Obviously this must be the subject of further investigations.

Since intravenously injected fluorescent albumin cannot enter into the endoneurial compartment of peripheral nerves in normal rats this species is well suited for further experiments on the behaviour of vascular permeability in experimentally induced peripheral neuropathies since even small differences can be detected. However, when the other species tested in the present study are to be used, the normally occurring endoneurial infiltration of serum albumin must be taken into consideration.

Summary

A preliminary study of the vascular permeability to serum albumin of peripheral nerves in different species is reported. The results show that species differences in the permeability of endoneurial blood vessels to circulating serum albumin exist in the peripheral nerves.

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